TITLE The Molecular Biology of Ammonia Assimilation in the Obligate Methanotroph *Methylococcus capsulatus* Strain Bath.

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The Molecular Biology of Ammonia Assimilation in the Obligate Methanotroph *Methylococcus capsulatus* Strain Bath.

by

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B.Sc. (Hons) (Warwick)

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DEDICATION

To my wife, Sue, whose encouragement and support throughout the duration of this project has made all this possible.

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Last, but not least, many thanks to Mrs. Carol Howes for her excellent typing and presentation of this manuscript.

Declaration

I declare that this thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself under the supervision of Dr. J. C. Murrell. All sources of information have been specifically acknowledged by means of reference.

Donald L. N. Cardy.

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Summary

The structural gene (*glnA*) encoding the ammonia assimilation enzyme glutamine synthetase (GS) has been cloned from the obligate methanotroph *Methylococcus capsulatus* (Bath). Complementation of *Escherichia coli* and *Klebsiella pneumoniae glnA* mutants was demonstrated. *In vitro* and *in vivo* expression analysis revealed the cloned *glnA* gene to encode a polypeptide with an apparent M_r of 60,000 as determined by PAGE.

Expression of the *M. capsulatus* (Bath) *glnA* gene in *E. coli* was found to be regulated by nitrogen levels in an Ntr^+ but not an Ntr^- background. This regulation was not observed when the cloned *M. capsulatus* (Bath) *glnA* gene was under the influence of the chloramphenicol acetyl transferase gene of the vector.

The nucleotide sequence of the *M. capsulatus* (Bath) *glnA* gene and flanking sequences has also been determined. The gene comprises 1407 bp encoding a polypeptide of M_r 51,717 containing 468 amino acids. The 5' leader region contains three putative promoters. Promoters P_1 and P_2 resemble the canonical -10 -35 *E. coli* type promoter. Promoter P_3 which is located between P_1 and P_2, resembles the NtrA dependant promoters of enteric organisms. A potential NtrC-binding site was also determined. The 3' flanking region contained a small putative open reading frame (ORF) encoding a polypeptide of M_r 7022. The identity of this polypeptide remains to be elucidated. Comparisons of *glnA* structural genes and GS enzymes at the nucleotide and amino acid levels between *M. capsulatus* (Bath) and both prokaryotes and eukaryotes have been determined.

The presence of *ntrA*, *ntrB*, *ntrC*, *glnB* and *rpoD* homologues in the *M. capsulatus* (Bath) genome was determined by heterologous hybridization studies. Type I and Type II obligate methanotrophs were also screened for *glnA*, *ntrC* and *ntrA* homologues. Both Type I and Type II organisms were found to have homologues to each of these gene probes.

A portion of a *M. capsulatus* (Bath) putative *ntrC* gene has been cloned on a cosmid, pCOS1 and was found to be unlinked to *glnA* and lies some 8.5 kb downstream of *glnA*.

The development of a plasmid transformation and gene transfer system for *M. capsulatus* (Bath) based on previously published methods has also been assessed.

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ABBREVIATIONS

A	Absorbance
ADH	Alanine dehydrogenase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMS	Ammonia mineral salts
Ap	Ampicillin sodium salt
ATP	Adenosine triphosphate
bp	Base pairs
Ci	Curie
Cm	Chloramphenicol
CO_2	Carbon dioxide
CPM	Counts per minute
dCTP	Deoxy-cytidine triphosphate
dGTP	Deoxy-guanosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenadiaminetetra-acetic acid
ESR	Electron spin resonance
FAD	Flavin adenine dinucleotide
FBP	Fructose-1-6-bisphosphate
FMP	3-fructose-6-phosphate
g	gramme
xg	gravitational force
GDH	Glutamate dehydrogenase
GOGAT	Glutamate synthase
GS	Glutamine synthetase

h	hour
Km	Kanamycin
kb	Kilobase
KDPG	2-keto-3 deoxy-6-phosphogluconate
L	Litre
M9	Mineral salts medium
mA	Milliamps (current)
mg	Milligram
µg	Microgram
min	Minute
MMO	Methane monooxygenase
M_r	Relative molecular mass
Ms	Mineral salts
NAD	Nicotinoamide adenine dinucleotide
NADP	Nicotinoamide adenine dinucleotide phosphate
NADH	Reduced form of NAD
NADPH	Reduced form of NADP
nm	Nanometre
NMS	Nitrate mineral salts
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
Pi	Inorganic phosphate
pO_2	Partial pressure of oxygen
PPO	2,5-diphenyloxazole
rpm	Revolutions per minute
RuMP	Ribulose monophosphate
SDS	Sodium dodecyl sulphate

sec	Second
Sm	Streptomycin
Sp	Spectinomycin
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
Tc	Tetracycline
TCA	Tricarboxylic acid
Tn	Transposon
Tris	Tris-hydroxymethylaminomethane
UV	Ultraviolet radiation
v/v	Volume/volume
w/v	Weight/volume

1. Introduction

1:1 General Introduction.

Methylococcus capsulatus strain Bath is a Gram negative, aerobic coccus which grows on methane as sole source of carbon and energy. This organism belongs to a group of organisms known as the C_1-utilizers, which are recognized by their ability to use compounds which are more reduced than carbon dioxide and contain no carbon-carbon bonds as sole carbon source for growth e.g. methane, methanol, N-methyl and S-methyl compounds.

C_1-utilizers can fall into one of two categories:-

i) The methylotrophs, defined by Colby and Zatman (1973), and Quayle and Ferenci (1978), as those organisms that obtain their energy from the oxidation of C_1-compounds and assimilate carbon as formaldehyde or a mixture of formaldehyde and carbon dioxide (CO_2).

ii) C_1-utilizing autotrophs, defined by Schlegel (1975), and Quayle and Ferenci (1978), as organisms that oxidize C_1 growth substrates to CO_2 (which in most cases provides energy for growth) and then assimilate the CO_2 formed.

Whittenbury and Kelly (1977) revised and expanded the term "autotrophy" of Schlegel (1975) to incorporate anaerobic methane producers, acetate producers and ammonia oxidizers with the methylotrophs and other C_1-utilizing organisms. Their definition of autotrophy encompasses all organisms which can synthesize all their cellular constituents from one or more C_1-compounds. These organisms can be further subdivided into 3 main groups, based on their C_1-compound assimilation pathways, i.e. (a) those possessing the ribulose monophosphate pathway (RuMP pathway); (b) those

possessing the serine pathway; (c) those possessing the ribulose bisphosphate pathway (RuBP pathway). Research carried out by Taylor (1977) on *Methylococcus capsulatus* (Bath) indicated that some C_1-utilizers might use both methylotrophic and autotrophic modes of metabolism simultaneously (see Whittenbury, 1980). However, in the absence of further evidence to the contrary, other C_1-utilizers are assumed to fall into one or other of the above two categories.

It is not the intention here to give a comprehensive review of the physiology and biochemistry of the C_1-utilizing organisms, the reader is referred to a number of recent reviews; (Colby *et al.*, 1979; Higgins *et al.*, 1981; Anthony, 1982).

1:2 Occurrence and historical perspective to the isolation of methanotrophs.

Methane is the most abundant reduced carbon compound in nature. Ehhalt (1976) estimated that between 500-800 million tons of methane are produced per year and released into the atmosphere as an end product of anaerobic microbial degradation of organic material. This is matched by an equal amount produced from natural gas wells. Clearly, methane represents an almost inexhaustible carbon and energy substrate for bacterial growth as it is chemically stable and there is a large amount of energy to be gleaned consequent on its oxidation. It is not surprising therefore, that a number of organisms have developed the ability to utilize this compound.

The occurrence and distribution of these organisms is widespread. In most habitats where methane diffuses into an aerobic environment, populations of methanotrophs can be found; e.g. soils, surface layers of sediments and natural waters. In aquatic environments, these microbes are most numerous in regions where methane, produced from the anaerobic decomposition of organic matter, and oxygen from the atmosphere, are

present at concentrations optimal for their growth (Hanson, 1980). The production and oxidation of methane in aquatic environments has been comprehensively covered in the review by Rudd and Taylor (1980).

The widespread occurrence and distribution of methane oxidizing bacteria was not realized until 1969-1970, when Whittenbury and his colleagues dramatically transformed the study of methane oxidizing bacteria by devising simple and effective techniques for enrichment and isolation of these microorganisms (Whittenbury, 1969; Whittenbury *et al.*, 1970(a)). Prior to this, only a handful of methane oxidizing bacteria had been isolated in pure culture. The first well characterized methane utilizing bacterium was isolated in pure culture in 1906 by Söhngen (Söhngen, 1906) and named, by him, *Bacillus methanica*. Relatively few additions were made to the list of known bacteria with these growth characteristics in the succeeding 50 years. In 1956, Dworkin and Foster re-isolated *Bacillus methanica* and renamed it *Pseudomonas methanica* (Dworkin & Foster, 1956). In the succeeding decade only three other new species of methanotroph were isolated and described in any detail (see Anthony, 1982).

The subsequent isolation of over 100 methanotrophs from mud, water and soil samples obtained worldwide by Whittenbury and his colleagues (Whittenbury *et al*, 1970(a)) using new enrichment, isolation and culture methods highlighted the apparent difficulty in isolating pure cultures of methanotrophs prior to this work. Since then, there have been a number of reports concerning the isolation and characterization of methanotrophs (Hazeu and Steenis, 1970; Malashenko *et al.*, 1972; Hazeu, 1975; Malashenko, 1976; Trotsenko, 1976; Galchenko, 1977; Hazeu *et al.*, 1980).

Patt *et al.* (1974) reported the isolation of a facultative methanotroph from Lake Mendota, Wisconsin, USA, which was able to grow on multicarbon substrates such as glucose, ethanol, acetate and succinate as well as methane and methanol. They named the organism *Methylobacterium organophilum* XX. Reports by Patt *et al.* (1974) and Patel *et al.* (1978)

described similar organisms named as strains of the new genus *Methanobacterium*. *Methylobacterium organophilum* XX unfortunately lost the ability to grow on methane after subculture on methanol or multicarbon substrates, possibly due to loss of a plasmid encoding the methane monooxygenase enzyme. This view was strengthened by the observation that a plasmid of high M_r was present in cells grown on methane (Haber *et al.*, 1983; Hanson, 1980), but could not be isolated from cells grown on other substrates. The isolation of other facultative methanotrophs have also been reported by other workers. Lynch *et al.*, (1980) described the isolation and characterization of two facultative methanotrophs; *Methylobacterium athanolicum* and *Methylobacterium hypolimneticum*. A recent report by Zhao and Hanson, (1984) indicated that a third type of methanotroph had been isolated which, although obligate in its requirements for methane or methanol, can be grown in the presence of 0.8% glucose. Under these conditions, up to 84% of cellular carbon could be derived from the added glucose.

The potential importance for genetic studies on these organisms was realized upon their isolation. One major advantage that these organisms offer over the obligate methanotrophs in the study of enzyme regulation relates to their mutability. O'Connor & Hanson, (1977, 1978) have noted that it is possible to obtain mutagenization of these organisms growing on glucose and other organic substrates by techniques which do not lead to the successful isolation of mutants of these strains and obligate methanotrophs, when growing on methane. This, therefore, presented the first good opportunity for extensive genetic and enzyme regulation studies on methane-oxidizing bacteria. However, the existence of facultative methanotrophs was seriously questioned following a report by Lidstrom-O'Connor *et al.*, (1983) on their own previously published isolate '*Methylobacterium athanolicum*' M414. This isolate has now been shown to consist of a stable mixture of two methylotrophs; one is an obligate

methanotroph (Strain POC) and is similar to *'Methylocystis'* species, as described by Whittenbury *et al.* (1970(b)); the other is a *Xanthobacter* species (Strain H414) which is a diazotroph and grows on $H_2 + CO_2$, methanol and multicarbon substrates. The POC strain was also shown to contain three cryptic plasmids which were present only in this member of the consortium. Since the methanotroph would be present only in very low numbers on methanol or multicarbon substrates, this may explain why plasmids appeared in the *M. organophilum* XX strain when grown on methane but not when grown on other carbon compounds. The authenticity of other 'pure' facultative methanotrophs must therefore, await independent verification.

1.3 Classification of obligate Methanotrophs.

One of the main consequences of the isolation of over 100 strains of methanotroph by Whittenbury and his colleagues was that, a simple classification scheme was proposed which divided the methanotrophs into two different types designated Type I and Type II, based largely on internal membrane arrangements (resembling those found in nitrifying and photosynthetic bacteria) and cell shape (Davies and Whittenbury, 1970). The complex internal membrane structure present in methanotrophs results in a surface area 4-8 times that of the cytoplasmic membrane. Type I bacteria have bundles of disc-shaped vesicles which appear to be formed by invagination of the cytoplasmic membrane, while Type II bacteria have a system of paired membranes situated around the periphery of the cell. Other workers (Smith *et al*, 1970; Smith and Ribbons, 1970) also noted these complex membrane arrangements in a number of methanotroph species, confirmed by the detailed freeze-etching studies of Weaver and Duggan (1975).

The role of these complex internal membranes has been the subject of speculation (Procter *et al*, 1969; Davies and Whittenbury, 1970; Higgins *et*

al, 1981). Similar membrane systems are found in photosynthetic bacteria, ammonia and nitrite oxidizers, cyanobacteria and some higher hydrocarbon utilizers all of which have special electron transport requirements, therefore it is probable that they are associated with peculiar oxidative properties of methanotrophs. As methylotrophs, able to grow on methanol but not methane, do not possess internal membranes, then a reasonable hypothesis would be that these membranes perform some special function in the initial step in methane hydroxylation. Higgins, (1979) proposed that the membranes may merely act by anchoring the oxygenase components.

The classification scheme proposed by Whittenbury and his colleagues for their isolates (Whittenbury *et al*, 1970(a)) divided the methanotrophs into five groups: *'Methylococcus'*, *'Methylomonas'* and *'Methylobacter'* (which possessed a Type I internal membrane arrangement), *'Methylosinus'* and *'Methylocystis'* (which possessed a Type II internal membrane arrangement). These five groups of methanotroph were based on other divergent properties between the two types e.g. the nature of the resting stage produced by the organism (Whittenbury *et al*, 1970(b)), cell and colony morphology. The five main groups of methanotroph were further subdivided into sub-groups on the basis of their physiological and biochemical properties (Whittenbury *et al*, 1970(a) (see Table 1:1).

Lawrence and Quayle (1970) discovered a correlation between the carbon assimilaton pathways and the intracytoplasmic membrane arrangements of a number of methanotrophs and Davey *et al*. (1972) found further biochemical differences between Type I and Type II organisms. This evidence gave further support to the division of the methanotrophs into two groups. On the basis of the above observations, Whittenbury and his colleagues constructed a tentative classification scheme for the methanotrophs (Whittenbury *et al*., 1976). This scheme has been revised to include facultative methanotrophs (Colby, Dalton and Whittenbury, 1979).

Table 1.1 Tentative classification scheme for methane-oxidising bacteria[a].

Determinants	Type I		Type II	
Membrane arrangement	Bundles of vesicular disks		Paired membranes around cell periphery	
Resting stages	Cysts (*Azotobacter*-like)		Exospores or lipid cysts	
Major carbon assimilation pathway	RuMP (hexulosephosphate synthase +)		Serine pathway (hydroxypyruvate reductase +; hexulosephosphate synthase -)	
TCA cycle	Incomplete (2-oxoglutarate dehydrogenase negative)		Complete	
Nitrogenase	-		+	
Predominant fatty acid C chain length	16		18	
	Subgroup A	Subgroup B	Subgroup obligate[b]	Subgroup facultative[c]
Presence of RuBP carboxylase	-	+	-	
DNA base ratio (%G+C)	50-54	62.5	62.5+ (where tested)	
Isocitrate dehydrogenase	NAD- or NADP-dependent	NAD-dependent	NADP-dependent	
Cell shape	Rod and ? coccus	Coccus	Rod and vibrio	
Growth at 45°C	Some +	+	-	
Presence of glutamate dehydrogenase when grown on ammonia	Present	Absent (uses alanine dehydrogenase)	Absent (uses GS/GOGAT)	
Examples	*Methylomonas methanica* and *Methylomonas albus*	*Methylococcus capsulatus*	*Methanomonas methanooxidans*, *Methylosinus trichosporium* (both obligate) and *Methylobacterium organophilum* (facultative)	

[a] Not all strains classifiable into Type I and Type II have been shown to possess all the biochemical characteristics outlined in this scheme.

[b] Use methanol and formaldehyde as carbon and energy source, but not C_2+ compounds.

[c] Use variety of organic compounds, e.g. glucose as carbon and energy source.

Taken from Dalton and Lask, 1985.

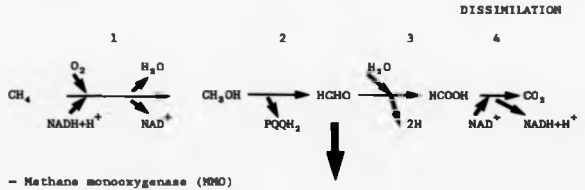
Since the scheme was proposed by Whittenbury and his colleagues, several anomalies have been detected, for instance, *Methylococcus capsulatus* (Bath) possesses more than one C_1-assimilation pathway, in addition to the ribulose monophosphate pathway. Key enzymes of the serine pathway and ribulose bisphosphate carboxylase exist in this organism (Taylor, 1977). *Methylococcus capsulatus* (Bath) is an organism which represents a third major type of methanotroph and has therefore been placed in a group known as the type X methanotrophs (Whittenbury and Dalton, 1981). The present position on nomenclature and taxonomy is confused and has quite rightly been described as 'chaotic' (Whittenbury and Krieg, 1984). A broad division into Types I, II and X is now generally accepted (Whittenbury *et al*, 1970(a); Taylor *et al*, 1981).

1:4 Physiology and biochemistry of obligate methanotrophs.

1:4:1 Carbon assimilation pathways.

a) Methane oxidation.

The oxidation of methane to carbon dioxide appears to proceed via a series of two electron oxidation steps. Formaldehyde occupies a central position in the metabolism of methane, since, it is at this level that the carbon is both assimilated into biomass and dissimilated to carbon dioxide to provide energy for growth. Both assimilatory and dissimilatory reactions, which are shown below, occur simultaneously in the cell.



1 – Methane monooxygenase (MMO)

2 – Methanol dehydrogenase (MDH)

3 – Formaldehyde dehydrogenase

4 – Formate dehydrogenase

Taken from Anthony, (1986).

The evidence for this pathway has been extensively reviewed by several workers (Anthony, 1982; Dalton and Leak, 1985; Anthony, 1986).

A brief summary on the bacterial oxidation of methane will be given in this section.

The first report of a methane oxidizing cell-free system was by Ribbons and Michalover (1970) who showed methane-stimulated respiration and methane stimulated NADH oxidation with particulate (membrane) preparations of *Methylococcus capsulatus* (Texas). Using a substrate analogue of methane, bromomethane, Colby *et al.*, (1975) demonstrated the NADH dependant disappearance of bromomethane in a particulate cell-free extract of *Methylomonas methanica*. Subsequently, there were reports of cell-free methane oxidizing activity in *Methylosinus trichosporium* (Tonge *et al.*, 1975) and *Methylococcus capsulatus* (Bath) (Colby and Dalton, 1976). It is largely from the work with these two organisms that methane oxidation has been elucidated and the methane oxidizing complex purified.

Both *M. trichosporium* and *M. capsulatus* (Bath) are now known to produce two types of methane monooxygenase (MMO) - the enzyme which catalyses the formation of methanol from methane. Both forms are able to use NADH as an electron donor *in vitro*. The particulate (membrane bound) forms of MMO are produced in conditions of copper sufficiency, and soluble types of MMO are formed when bacteria are grown under conditions of copper limitation. Copper sufficiency and insufficiency are determined by the copper concentration in the growth medium and by cell density, and so they can appear to be determined by alterations in the growth rate or in the carbon, oxygen or nitrogen supply (Dalton *et al.*, 1984; Prior and Dalton, 1985).

An excellent review which covers work carried out on these methane monooxygenases has recently been published (Anthony, 1986) and therefore will not be described in any detail here. However, a brief description of the work carried out on the soluble MMO from *M. capsulatus* (Bath) will be outlined.

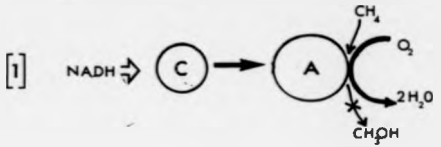
The soluble MMO from *M. capsulatus* (Bath) is induced during growth under copper insufficiency (Stanley *et al.*, 1983; Dalton *et al.*, 1984). The complex exists free in solution and has been resolved into three fractions, A, B and C by DEAE ion exchange chromatography (Colby and Dalton, 1978). All three components of the soluble MMO from *M. capsulatus* (Bath) have now been purified and characterized (Dalton and Leak, 1985). Protein A has a relative molecular mass (M_r) of 210,000 and is composed of three subunits α, β and γ of which there are two copies of each. α, β and γ are of M_r 54,000, 42,000 and 17,000 respectively. It is an acidic protein which contains two atoms of non-haem iron per molecule and a small amount of zinc as measured by atomic adsorption spectroscopy. No acid-labile sulphur was detected (Woodland and Dalton, 1984(a)). Protein A is thought to be responsible for substrate binding. A large change in the electron spin resonance (ESR) spectrum of reduced protein A was observed in

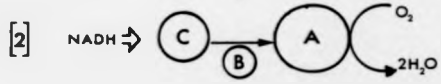
the presence of a substrate (Woodland and Cammack, 1985).

Protein B, is a colourless protein consisting of a single polypeptide chain of M_r 16,000. It is devoid of prosthetic groups and like protein A has no discernible independant catalytic activity (Green and Dalton, 1985). Protein B is regarded as a powerful regulator of enzyme activity converting soluble methane monooxygenase, from an oxygenase, to an oxidase, in response to environmental changes.

Protein C is an iron-sulphur flavoprotein, consisting of a single polypeptide of M_r 42,000, containing one molecule of flavin adenine dinucleotide (FAD), two atoms of iron and two atoms of acid-labile sulphide (Colby and Dalton, 1978, 1979; Lund, 1983). The iron and sulphide are present as a single iron-sulphide centre of the $[2Fe\text{-}2S^{*}\ (S\text{-}Cys)_4]$ type determined by core extrusion and ESR studies (Lund and Dalton, 1985). Protein C, unlike proteins A and B, has a measurable independant activity as an NADH-acceptor reductase activity i.e. it catalyses the transfer of electrons from NADH to a variety of electron acceptors, such as potassium ferricyanide, oxygen and protein A.

In the presence of protein B, protein C can pass electrons singularly from NADH, donating them to protein A at a constant redox potential. These electrons can then be used to reduce oxygen to water resulting in an NADH:oxidase activity for protein A plus protein C complex (Lund *et al.*, 1985). Electron flow in this case is independent of the presence of protein B, however, protein B does act to shutdown this electron flow in the absence of a oxidizable substrate, such as methane (Green and Dalton, 1985). The addition of substrate to proteins A and C has no effect on the flow of electrons. Protein B has been postulated to act to couple the flow of electrons from NADH through protein C to protein A, to the oxidation of substrate, switching the enzyme complex from an oxidase to an oxygenase (Green and Dalton, 1985). A summary of the mechanism of the soluble MMO from *M. capsulatus* (Bath) is given in Figure 1:1.


[1]
NADH
C
A
CH_4
O_2
$2H_2O$
CH_3OH


[2]
NADH
C
B
A
O_2
$2H_2O$

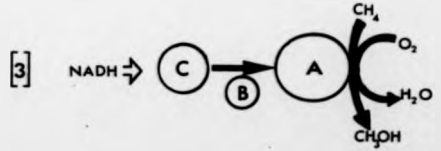

[3]
NADH
C
B
A
CH_4
O_2
H_2O
CH_3OH

Figure 1:1 Mechanism of soluble MMO.

[1] Proteins A and C catalyze the novel four electron reduction of oxygen to water in the presence or absence of a hydroxylatable substrate, for example CH_4.

[2] Addition of protein B switches the enzyme complex from an oxidase to an oxygenase and the reduction of oxygen to water is no longer catalyzed. In the absence of CH_4, steady-state electron transfer between proteins A and C is shutdown.

[3] Addition of CH_4 to the complete methane monooxygenase complex restores inter-protein electron transfer and the oxygenase reaction is catalyzed to the complete exclusion of the oxidase reaction. The width of the arrows in the diagram reflect the relative rate of the reaction indicated.

Taken from Green and Dalton, (1985).

In addition to methane, a wide variety of other substrates have been shown to be oxygenated by the MMO complex from the obligate methanotrophs. They include alkanes, alkenes, dimethyl- and diethyl-ether, alicyclic, aromatic and heterocyclic compounds and ammonia (Colby *et al.*, 1977; Stirling *et al.*, 1979; Higgins *et al.*, 1979).

b) Methanol oxidation.

Studies carried out on a number of methane oxidizing bacteria have shown the presence of a broad specificity NAD(P)-independent methanol dehydrogenase (reviewed in Anthony, 1986). The properties of this enzyme were described originally in *Pseudomonas* M27 (Anthony and Zatman, 1964). Typically, methanol dehydrogenases oxidize a wide range of primary alcohols using phenazine methosulphate as an artificial electron acceptor and ammonia or methylamine as activator. They are usually dimers of identical subunit M_r of 60,000. *In vivo* the enzyme is thought to be coupled to the electron transport chain at the level of cytochrome C (Duine and Frank, 1981). Recently, this activity has been demonstrated *in vitro* using anaerobically prepared enzyme and cytochrome C (Beardmore-Gray *et al.*, 1983).

Until recently the nature of the prosthetic group of methanol dehydrogenase had not been determined, although it has now been established that methanol dehydrogenase is a quinoprotein with a pyrrolo quinoline quinone (PQQ) prosthetic group (Duine and Frank, 1980) as have been found in a number of other dehydrogenases e.g. glucose dehydrogenase (Duine *et al.*, 1979).

c) Formaldehyde and formate oxidation.

Formaldehyde produced by oxidation of methanol or methylamine can either be assimilated into cell material or dissimilated by complete oxidation to CO_2. This oxidation of formaldehyde to CO_2 can occur by two routes in C_1-utilizing organisms: 1) dehydrogenation to formate and thence to CO_2 by formate dehydrogenase, 2) oxidized to CO_2 by a cyclic enzyme scheme involving hexulose phosphate synthase. The former route involves the enzyme formaldehyde dehydrogenase of which two forms exist, an NAD(P)-linked and a NAD(P)-independent form. An NAD(P) linked formaldehyde dehydrogenase was purified from *Methylococcus capsulatus* (Bath) which is a dimer of equal subunit M_r of 57,000 (Stirling and Dalton, 1978). The formate so formed, is further oxidized by a NAD-linked formate dehydrogenase to CO_2 (Stirling and Dalton, 1978).

The latter route is via a cyclic series of reactions involving hexulose phosphate synthase which yields CO_2 and two molecules of NAD(P)H, was first proposed by Strøm *et al.* (1974) working with *Methylococcus capsulatus* (Texas) and *Methylomonas methanica* and also by Colby and Zatman (1975).

Methanol dehydrogenase may have a major role in formaldehyde oxidation in the methanotrophs due to its dual specificity for methanol or formaldehyde *in vitro*. Whether or not this enzyme functions *in vivo*, in this way, is not known.

d) Formaldehyde fixation pathways.

Carbon for the biosynthesis of cellular material is diverted from the methane oxidation pathway at the oxidation stage of formaldehyde and is assimilated either via a ribulose monophosphate (RuMP) pathway or a serine pathway. The pathway used, can be correlated with the type of membrane

system observed, i.e. Type I organisms use the RuMP pathway and Type II organisms use the serine pathway. The biochemistry of the methanotroph carbon assimilation pathways has been extensively reviewed (Quayle, 1980; Higgins *et al.*, 1981; Anthony, 1982), and this aspect of methanotroph biochemistry is not directly relevant to the work contained in this thesis, it will not be considered in detail but will be briefly summarized here.

i) The ribulose monophosphate pathway (RuMP).

The first intimation that carbon was not assimilated at the level of CO_2 was obtained during studies carried out on *Methylomonas methanica* by Leadbetter and Foster (1958) and by Johnson and Quayle (1965) who showed that ribulose bisphosphate carboxylase, the key enzyme in the ribulose bisphosphate pathway of CO_2 fixation was absent from this organism. Subsequent studies into the pathways of carbon assimilation in the obligate methanotroph *Methylomonas methanica* by Kemp and Quayle (1967) enabled them to propose the ribulose monophosphate pathway of formaldehyde assimilation.

In the RuMP pathways all the cell carbon is assimilated at the oxidation level of formaldehyde. The overall reaction cycle synthesises one molecule of a C_3 compound from three molecules of formaldehyde, this C_3 compound being either pyruvate or dihydroxyacetone phosphate. The RuMP pathway may be conveniently divided into three stages. Stage 1 of the cyclic sequence (fixation) is the aldol condensation of formaldehyde with three molecules of ribulose-5-phosphate to yield 3-hexulose-6-phosphate (hexulose phosphate), which is then isomerized to 3-fructose-6-phosphate (FMP); Stage 2 of the cycle (cleavage), one of the FMP molecules is converted to either fructose-1-6-bisphosphate (FBP) by phosphofructokinase, or to 2-keto 3-deoxy 6-phosphogluconate (KDPG) by the Entner/Doudoroff enzymes; these molecules are then cleaved by aldolases to glyceraldehyde 3-phosphate plus the "product" of the pathway, which is either, pyruvate

(from KDPG) or dihydroxyacetone phosphate (from FBP); The final stage of the cycle (rearrangement) involves the regeneration of three molecules of ribulose 5-phosphate from the two molecules of fructose 6-phosphate and one molecule of glyceraldehyde phosphate produced in the fixation and cleavage steps (stages 1 and 2 respectively). These sugar phosphate interconversions are catalyzed by transaldolase and transketolase in methanotrophs (Strøm *et al.*, 1974).

ii) The serine pathway.

Much of the earlier work which led to the derivation of the serine pathway was carried out by Quayle and his colleagues using the facultative methanol and formate utilizers, as these organisms, due to their versatility of growth substrates, are considerably easier to work with (see Anthony, 1982).

The key intermediates in this pathway of formaldehyde fixation are carboxylic acids and amino acids, as opposed to the phosphorylated sugars in the RuMP pathway. In this pathway, part of the cell carbon arises from CO_2 by activity of phosphoenol pyruvate carboxylase and the rest from activity of serine transhydroxymethylase which catalyses serine synthesis from glycine and formaldehyde. There are two variants of the serine pathway which differ in the method of regeneration of glycine depending on the presence or absence of isocitrate lyase (icl). In icl^+ organisms, glyoxylate is regenerated by the oxidation of acetyl CoA; in icl^- organisms, glyoxylate is regenerated by the homoisocitrate-glyoxylate cycle (see Kertsen 1980, 1981). The final part of the pathway is the conversion of acetyl-CoA to the C_3 and C_4 skeletons required for biosynthesis of cellular material.

Very little work has been done on the complete pathway in methanotrophs and much evidence for its existence in the Type II organisms

has relied upon the identification of one or two of the key enzymes i.e. serine transhydroxymethylase, malyl-CoA lyase, hydroxypyruvate reductase and serine-glyoxylate aminotransferase (see Lawrence and Quayle, 1970; Malashenko, 1976; Trotsenko, 1976).

iii) The ribulose bisphosphate pathway of carbon dioxide fixation.

Taylor (1977) made the first observation of the presence of ribulose bisphosphate carboxylase and phosphoribulokinase, key enzymes of the Benson-Calvin cycle for CO_2 fixation, in the methanotroph *M. capsulatus* (Bath). This was an important and indeed surprising finding as *M. capsulatus* (Bath) was thought to assimilate most of its cell carbon by way of the RuMP pathway of formaldehyde fixation. $^{14}CO_2$ experiments in this organism confirmed that the RuBP carboxylase was operating *in vivo* (Taylor, 1981) and it was concluded that only about 2.5% of cell carbon arose from CO_2 during growth on methane as carbon substrate. Approximately 75% of this was attributable to conventional carboxylation reactions and therefore it was concluded that RuBP carboxylase was of little physiological significance in this organism. Stanley and Dalton (1982) investigated the role played by RuBP carboxylase and phosphoribulokinase in *M. capsulatus* (Bath) and found that the combined action of these enzymes produced 2 moles of 3-phosphoglycerate (PGA) from 1 mole of RuMP. One mole of PGA was used for the synthesis of glyceraldehyde 3-phosphate to replenish the arrangement reactions and the other mole of PGA was used for the synthesis of the glycolysis components. In *M. capsulatus* (Bath) FBP aldolase is absent, therefore the above satisfies the demand for glyceraldehyde 3-phosphate and the generation of glycolytic intermediates. All other Type I organisms which lacked RuBP carboxylase had significant levels of FBP aldolase and presumably used that route for the synthesis of C_3 intermediates. Dalton and Leak (1985) suggested that as *M. capsulatus*

(Bath) does not grow autotrophically (Stanley and Dalton, 1982) that this organism may represent an intermediate form between the primitive RuMP-type organisms and the chemolithotrophs such as *Nitrosomonas* (which also oxidizes methane, (Hyman and Wood, 1983) and have an incomplete TCA cycle).

1:4:2 Nitrogen metabolism in the obligate methanotrophs

Obligate methanotrophs can utilise both nitrate and ammonia as nitrogen sources and some can also grow diazotrophically.

1:4:2:1 Nitrogen fixation.

Since the first report of nitrogen fixation in methane oxidizing bacteria by Davis *et al.* (1964), very few reports of dinitrogen fixation in methanotrophs have been published. However, with the advent in 1966 of a relatively cheap and rapid technique for assaying nitrogenase activity being developed, several reports of methane oxidizing bacteria which fix N_2 have appeared. This assay utilizes the ability of nitrogenase to reduce acetylene to ethylene and is known as the acetylene reduction test (Dilworth, 1966; Schöllhorn and Burris, 1967).

de Bont and Mulder (1974) carried out a detailed study of N_2-fixation on a methanotroph which closely resembled *Methylosinus sporium* (Whittenbury *et al*, 1970(a)). They found that the growth of this organism was oxygen sensitive when grown in nitrogen-free mineral salts medium under a methane/air atmosphere. If the partial pressure of oxygen was reduced, growth was markedly increased. This oxygen sensitivity was due to the inhibitory effect of oxygen on the nitrogenase enzyme, a phenomenon which had previously been observed in *Azotobacter chroococcum* (Dalton and Postgate, 1969). Also, de Bont and Mulder noted that the acetylene reduction test could only be carried out on methanol grown cells and not

with methane grown cells. This phenomenon they attributed to possible co-oxidation of the ethylene or an inhibitory effect of acetylene on methane oxidation.

Subsequent work carried out by Dalton and Whittenbury (1976) on *M. capsulatus* (Bath) showed that the failure of the acetylene reduction test in the presence of methane, was due to inhibition of methane oxidation by acetylene. They demonstrated that chemostat grown cells of *M. capsulatus* (Bath) growing on methane and fixing N_2 would actively reduce acetylene in sealed flasks at a lowered pO_2 provided that a suitable electron donor other than methane (e.g. methanol, formaldehyde or formate) was available. Using this technique, de Bont, (1976) reported that several '*Methylomonas* type' and '*Methylosinus* type' methane oxidizers were capable of fixing N_2 although he found that the facultative organism *Methylobacterium organophilum* strain XX did not reduce acetylene.

A survey on a number of representative species of obligate methane-oxidizing bacteria for their ability to fix N_2 by growth experiments and the acetylene reduction test was reported by Murrell and Dalton (1983(a)). This work once again indicated a fundamental difference between Type I and Type II methanotrophs. Of all the strains tested, only Type II organisms and *M. capsulatus* (Bath) grew well in nitrogen-free liquid medium and were capable of active acetylene reduction. *M. capsulatus* (Bath) has a number of characteristics which separate it from other Type I methanotrophs. Its ability to fix nitrogen provides further support for its inclusion in a separate group, the so called, Type X methanotrophs (Whittenbury and Dalton, 1981).

Oxygen sensitivity of diazotrophic growth of these organisms was reported (Murrell and Dalton, 1983(a)), as described in the few methanotrophs previously shown to fix N_2 (de Bont and Mulder, 1974; Dalton and Whittenbury, 1976; Dalton, 1980). Type II methanotrophs were found to be less sensitive to O_2 than the Type X methanotroph *M. capsulatus* (Bath)

and batch cultures of Type II organisms could be established at pO_2 values above 0.2 bar. N_2 fixation in *M. capsulatus* (Bath) was found to be inhibited at pO_2 values above 0.15 bar and the 'switch off' of nitrogenase by ammonia, a phenomenon that has been described in detail for members of the Rhodospirillaceae (Kanamoto *et al.*, 1984) was also observed in this organism (Murrell and Dalton, 1983(a)).

1:4:2:2 Ammonia assimilation.

Ammonia assimilation has been extensively studied in the Enterobacteriaceae and several other microorganisms. This section is only concerned with reports on ammonia assimilation in the obligate methanotrophs and the reader is referred to Section 1:6 for a detailed review on ammonia assimilation in other organisms.

The first extensive survey of ammonia assimilation in methanotrophs was reported by Shishkina and Trotsenko (1979). Cell-free extracts obtained from *Methylomonas methanica* 12, *Methylosinus trichosporium* 44 and *Methylococcus capsulatus* (Foster and Davis strain), grown in medium containing ammonia, nitrate or no fixed nitrogen source, were examined for the presence of ammonia assimilating enzymes. These workers concluded that Type I methanotrophs assimilated ammonia via glutamate dehydrogenase (GDH) or alanine dehydrogenase (ADH) and glutamine synthetase (GS), but not via the GS/GOGAT pathway, as no glutamate synthase (GOGAT) activity was observed. The Type II methanotrophs, were proposed to assimilate ammonia exclusively via the GS/GOGAT pathway as no amino acid dehydrogenase activities were detected in cell-free extracts of *M. trichosporium*. In *M. capsulatus* (Foster and Davis strain) Shishkina and Trotsenko proposed that during growth on N_2, this organism utilized the GS/GOGAT pathway as well as ADH for the assimilation of ammonia. This result is somewhat surprising, since, in most organisms which possess ADH, the activities of this enzyme

are highest in cell-free extracts from ammonia grown cells.

Unfortunately, cell-free extracts used in this study, were obtained from batch grown cells, hence making it difficult to define exact growth conditions and to compare enzyme levels in different methanotrophs.

Up until 1983, very little research had been carried out on ammonia assimilation in methanotrophs. The most detailed study of ammonia assimilation has come from Murrell and Dalton (1983(b)), who studied the ammonia assimilation pathways of three obligate methanotrophs; the Type X organism, *Methylococcus capsulatus* (Bath), the Type I methanotroph, *Methylomonas methanica* S1 and the Type II methanotroph, *Methylosinus trichosporium*, OB3b. In this study, all the obligate methanotrophs were shown to assimilate ammonia via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Type II methanotrophs were found to assimilate ammonia exclusively via the GS/GOGAT pathway, whether grown on dinitrogen, nitrate or ammonia. Type I methanotrophs were found to possess other ammonia assimilation pathways. The pathway by which ammonia is assimilated in Type I methanotrophs is determined by the fixed nitrogen source present. In order to determine which ammonia assimilation pathways were operating in the Type II methanotrophs and the Type X methanotroph *M. capsulatus* (Bath), cell extracts were taken from ammonia, nitrate and dinitrogen grown steady state cells and assayed for ammonia assimilation enzymes. Murrell and Dalton (1983(b)) found that during growth on nitrate, Type I methanotrophs possessed high GS and GOGAT activity and little or no alanine dehydrogenase (ADH) or glutamate dehydrogenase (GDH) activity, suggesting that the GS/GOGAT pathway was the primary pathway of ammonia assimilation. Growth on ammonia however, showed repression of the GS/GOGAT pathway enzymes and ammonia was assimilated either by the ADH or GDH pathway.

Glutamine synthetase has been purified from the Type X organism *M. capsulatus* (Bath) (Murrell and Dalton, 1983(c)). The purified enzyme was reported to have a molecular weight of 617,000 and a subunit size of 60,000

Dal. The enzyme was shown to be partially regulated by cumulative feedback inhibition by several end products of glutamine metabolism. Control of glutamine synthetase activity by adenylylation/deadenylylation was demonstrated for the enzyme isolated from cells grown with different nitrogen sources i.e. glutamine synthetase isolated from N_2 grown cells was shown not to be adenylylated and was therefore fully biosynthetically active, whereas glutamine synthetase isolated from ammonia grown cells was fully adenylylated and therefore biosynthetically inactive.

1;4;2;3 Ammonia oxidation.

The first report of ammonia oxidation by a methanotroph was by Hutton and Zobell (1953), who reported the production of nitrite in media containing ammonia as the nitrogen source by an unidentified Type I methanotroph. Subsequent reports have shown that high concentrations of ammonia (NH_3 and NH_4^+) inhibit the growth of methanotrophs and competitively inhibit methane oxidation in these organisms. The most comprehensive work to date has been that of O'Neil and Wilkinson (1977) working with whole cells of *Methylosinus trichosporium* OB3b; and Dalton (1977); Pilkington (1983) working with cell-free extracts of *Methylococcus capsulatus* (Bath).

Dalton's studies of ammonia oxidation in cell-free extracts of *M. capsulatus* (Bath) (Dalton, 1977) showed that ammonia was first oxidized to hydroxylamine which was subsequently oxidized to nitrite. Dalton presented strong evidence in support of the hypothesis that the methane monooxygenase (MMO) was responsible for the initial oxidation step and based this on four lines of evidence:- i) Both ammonia and methane oxidation require the presence of reduced pyridine nucleotides and oxygen for activity. ii) The oxidation of methane to methanol and ammonia to hydroxylamine were specifically inhibited by acetylene, 8-hydroxyquinolene and methanol.

iii) A number of metal chelating agents had no inhibitory effects on either methane or ammonia oxidation. iv) Methane was a good inhibitor of ammonia oxidation and vice versa.

Pilkington (1983), using purified components of the soluble MMO of *M. capsulatus* (Bath), showed that ammonia oxidation was catalysed by the soluble MMO as well as presenting evidence which suggested that the particulate MMO also catalysed this reaction.

1:5 Genetics and molecular biology of the obligate methanotrophs.

1:5:1 Introduction.

Information on the genetic analysis and manipulation of a range of C_1-utilizing microorganisms is accumulating steadily (for reviews see Lidstrom, 1983; Holloway *et al.*, 1987). However, relatively few reports on the development and application of genetic techniques in the obligate methanotrophs have been forthcoming. The reason why this area has been largely neglected is due to the fact that obligate methanotrophs are particularly difficult organisms with which to carry out genetic studies. They are characterized by relatively long generation times (6-30 hours for most strains), they grow poorly on solid media (some require 2-4 weeks to grow on agar plates) and they have limited metabolic capabilities, with most growing on no substrate, other than methane.

1:5:2 Mutant isolation.

Perhaps the most difficult problem in developing genetics in this group of organisms, concerns mutant isolation. Harwood *et al.* (1972) and Williams *et al.* (1977) carried out a number of experiments using a variety of chemical and physical mutagens, such as; U.V., N-methyl-N-nitro-N-

nitrosoguanidine, N-nitroso-N-methyl urethane, ethyl methane sulphonate, methyl methane sulphonate, nitrous acid and γ-irradiation, none of which increased the frequency of spontaneous mutation (which was too low to allow the isolation of spontaneously occurring auxotrophs, Harwood *et al.*, 1972) in the methanotrophs. Williams *et al.*, (1977) postulated that the difficulty encountered in isolating stable methanotroph mutants is due to a lack of an error prone SOS repair system, hence, any mutants isolated revert at very high frequency. Due to this phenomenon, mutants for which no direct selection exists are extremely difficult to isolate. Since the spontaneous mutation frequency is in the order of 10^{-8}, even with the use of enrichment procedures, millions of colonies would have to be screened to detect any mutants (Harwood *et al.*, 1972). Apart from drug resistant mutants, the only stable mutant isolated in any obligate methanotroph was a leaky p-aminobenzoic acid requiring auxotroph of *Methylococcus capsulatus* (Foster and Davies, 1966), which arose spontaneously (Harwood *et al.*, 1972).

1:5:3 Plasmid screening of obligate methanotrophs.

Warner *et al.* (1977) carried out the first reported screening of methanotrophs for the presence of extra-chromosomal DNA. They screened three obligate methanotrophs; *Methylomonas methanica*, *Methylococcus capsulatus* (NCIB 11083) and *Methylosinus trichosporium* OB3b as well as two facultative methylotrophs; *Pseudomonas* AM1 and *Pseudomonas exotorquens* for plasmid DNA. They reported the isolation of three distinct plasmids from *Pseudomonas* AM1, but none were detected in the other species. The method employed in these studies appeared fairly harsh and may have resulted in shearing of large plasmids, thereby questioning the validity of results obtained in this study.

To bypass some of these problems inherent in using classical genetic techniques, Lidstrom and Wopat (1984) took a recombinant DNA approach to

genetic studies in the methanotrophs. They initiated their work by assessing the plasmid content of the methanotrophs with a view to studying possible plasmid-encoded functions. Plasmids which ranged in size from 27 to 125 megadaltons were isolated from nine out of ten methanotroph strains studied, the exception being *M. capsulatus* (Bath) (see Table 1:2). Restriction digest analysis of the plasmids from each strain revealed no distinctive pattern of common bands, which suggested they contained no large region of homology except for three strains of *Methylosinus trichosporium* which appeared to contain identical plasmids. Subsequent Southern hybridization analysis revealed that plasmid DNA isolated from *Methylosinus trichosporium* OB3b showed regions of homology with plasmid DNA isolated from *Methylosinus sporium* 5 and *Methylomonas albus*, but not with the plasmid DNA from the other obligate methanotrophs. Lidstrom and Wopat (1984) proposed that the function of these homologous regions although unknown, may reflect common replication regions or other genes. All of these plasmids are to date cryptic in nature and only one, from *Methylomonas albus* BG8, has been mapped (Lidstrom and Wopat, 1984).

Haber *et al.*, (1983) identified two plasmids of 130 and 210 kilobase pairs from a Type II obligate methanotroph, SB-1. The smaller of these plasmids, pR6-1 has been restriction endonuclease mapped and restriction fragments cloned into the cloning vector pBR322 and stably maintained in *Escherichia coli* HB101.

The nature of these plasmids with regard to conjugation is speculative since their cryptic nature does not allow selection of conjugal transfer. The transfer of chromosomal markers can be facilitated by some conjugative plasmids through mobilization of these markers during exchange of plasmid DNA between mating pairs. Matings in broth cultures between SB-1 and *Pseudomonas aeruginosa* auxotrophic mutants, were carried out in order to determine whether plasmids of methanotrophs could function in this manner. In one such mating with *P. aeruginosa* PAO 905 (a leucine auxotroph),

Table 1:2 Plasmids detected in methanotrophs.

Strain	Approximate Size (Mdal)
Type I	
Methylomonas albus BG8	25.6
Methylobacter capsulatus Y	63.2
Methylomonas methanica S1	125
Type X	
Methylococcus capsulatus	none detected
Type II	
Methylosinus trichosporium OB3b	125, 107, 97
Methylosinus trichosporium OB3bH	125, 107, 97
Methylosinus trichosporium OB5b	125, 107, 97
Methylosinus sporium 5	114.4, 72.2
Methylocystis parvus OBBP	125, 107
Methylocystis POC	118, 102, 50

Taken from Lidstrom *et al.*, (1984).

complementation to leucine protrophy was observed at a frequency of 2×10^{-4} Leu^{+} transconjugants per recipient cell. Plasmid DNA isolated from these transconjugants was found to be structurally different from pR6-1 and the resident plasmid in *P. aeruginosa* PAO 905, but shared sequence homology with each (Haber *et al.*, 1983). The pR6-1 DNA present in these transconjugants and the organism SB-1 DNA that complements the leucine mutation, have not yet been identified.

1.3.4 Development of gene transfer systems.

Some potentially useful gene transfer systems have been identified in some methanotrophs. Broad-host-range (Inc P1) conjugative plasmids have been transferred into several methanotrophs (Warner *et al.*, 1980; McPhaat *et al.*, 1987; Al-Taho and Warner, 1987). Inc W and Inc Q plasmids have also been transferred into the obligate methanotroph *Methylomonas albus* at low frequencies (McPhaat *et al.*, 1987). The Inc P plasmids R68.45, R751 and derivatives carrying bacteriophage Mu and/or transposons and pS-a (Inc W plasmids) were transferred to *M. albus* from *E. coli* at frequencies of 10^{-7}-10^{-8} per donor. The transfer frequencies reported from *E. coli* to *M. albus* for Mu-containing plasmids, indicated that the Mu-associated suicide phenomenon did not occur in *M. albus* and therefore cannot be used for the purpose of transposon mutagenesis in this organism. McPhaat *et al.*, (1987) suggested that due to the high frequency of interspecies transfer of RP4 and R300B, that this may allow the use of these two plasmids for genetic mapping in *M. albus* via the generation of R-primes, and also gene cloning studies.

In contrast, several studies have reported molecular cloning of genes from methanol utilizers (methylotrophs) and mobilisation of chromosomal markers between different strains, using broad host range plasmids; Haber *et al.* (1983); Tatra and Goodwin (1984); Al-Taho and Warner (1987); Kearney

and Holloway (1987). These studies have shown that constructed broad-host-range conjugative and mobilizable plasmid derivatives can serve as cloning vectors in methylotrophs. Encoding multiple drug resistances and possessing several unique cloning sites, these plasmids have enabled the construction of gene banks which can be maintained in *E. coli* and transferred to methylotrophs. The use of R-prime plasmids for the mobilization of chromosomal markers is an effective way to bypass *in vitro* recombinant DNA techniques. The application of such *in vivo* cloning methods to methylotrophs has provided a means for the identification of desired genes.

Lidstrom and her colleagues have now developed a conjugation system for gene transfer in three different methanotrophs (Lidstrom *et al.*, 1983). They used the Inc Pl cosmid cloning vector pVK100 (Knauf and Nester, 1982) which they found could be mobilized by the hybrid conjugative plasmid pRK2013 (Figurski and Helsinki, 1979) into three different methanotrophs. High frequencies of transfer (10^{-2}) were obtained for *Methylomonas albus* and *Methylocystis* FOC, sufficiently high to allow for direct complementation of mutants, whereas lower frequencies of transfer (10^{-4}) were obtained for *Methylosinus* 6, sufficient only for moving specific hybrid plasmid into cells. The development of this system could therefore allow specific genes to be cloned into this cosmid and be maintained in a variety of methanotrophs. The cosmid pVK100 is capable of carrying DNA fragments of from 15-30 kilobases in size, therefore providing a gene mapping tool for complementation analysis. The tools are now available to carry out gene transfer in the methanotrophs and the requirement for mutants is now therefore essential.

1:5:5 Mutagenesis

The isolation of mutants has been particularly difficult in the methanotrophs (see Sub-Section 1:5:2). Lidstrom *et al.* (1984) attempted to use transposon mutagenesis to facilitate mutant isolation. A variety of 'suicide' vehicles (plasmids which are not stably maintained in the recipient) carrying a variety of transposons were used including pJB4JI (Beringer *et al.*, 1978), a hybrid Inc P1-Mu-Tn5 plasmid, and several ColE1 plasmids containing Tn5, Tn10 and Tn7. None of these plasmids were successful in generating mutants.

Toukdarian and Lidstrom (1984) were successful however, in generating transposon mutants by the marker exchange technique (Ruvkun and Ausubel, 1981; Ruvkun *et al.*, 1982). A 2.3 kb *Hin*dIII region of the *Methylosinus* 6 chromosome was isolated from a gene library in pRR325, containing a portion of the structural genes for nitrogen fixation (*nif* genes). This fragment was mutagenized in *E. coli* using a defective lamda-Tn5 phage, and each unique transposon insertion was moved back into the chromosome of M.6 by the following one-step marker exchange technique. The mutagenized M.6 fragment was cloned into pRR325, which is unable to replicate in M.6, and was mobilized into this strain by pRK2013. After mating the Tn5-carrying plasmids into M.6, transconjugants were selected for the transposon drug marker (in this case, kanamycin). Each of the kanamycin-resistant clones generated in this way was shown to be unable to grow without a source of fixed nitrogen and unable to fix atmospheric nitrogen. The mutants generated in this way were found to be stable, and have been shown to be missing three *nif*-specific polypeptides. DNA hybridization analysis of these *nif* mutants showed that, although one out of five had apparently been produced as a result of a double-crossover recombination event, a variety of molecular events had led to the production of the other four mutants. Hence, since the molecular events leading to the generation of the mutants

tended to be complex, each mutant required characterization at the molecular level. Toukdarian and Lidstrom (1984) have shown that the technique of marker exchange can be used to generate straightforward, stable mutants in obligate methanotrophs provided that the resultant mutants are screened for double-crossover events. Successful one-step marker exchange represents a major accomplishment in the genetic manipulation of these organisms, no other mutagenesis procedure has been similarly successful.

Recently, Nicolaidis and Sargent reported the isolation of stable mutants deficient in MMO from *Methylosinus trichosporium* (OB3b). The selection procedure involved the use of dichloromethane which in 'wild type' cells is co-oxidised by MMO into carbon monoxide creating lethal conditions for 'wild type' MMO^+ strains. This method thus enables the direct selection for MMO^- mutants, but relies on the ability of the methanotroph to sustain growth on methanol as the carbon source. Four mutant strains were isolated, using this method, which showed a complete and irreversible elimination of MMO enzymatic activity (Nicolaidis and Sargent, 1987). A similar approach was utilized by McPheat *et al.* (1987), who successfully isolated stable MMO^- *Methylomonas albus* BG8WM mutants using resistance to dichloromethane for their selection.

1.3.6 **Transformation and [illegible]**

Genetic transformation with linear DNA has been described for only two methane oxidizing bacteria; the obligate methanotroph *Methylococcus capsulatus*, Foster and Davis strain (Williams and Rainbridge, 1971) and the facultative methyletroph, *Methylobacterium organophilum* XX (O'Connor *et al.*, 1977), but large amounts of DNA and long contact times were required in both cases.

Methanotroph bacteriophages have been isolated (Tyutikov *et al.*, 1980; Tyutikov *et al.*, 1983). 23 bacteriophage were isolated from 16 out of 88

studied samples (underground waters, pond water, soil, gas and oil installation waters, fermenter cultural fluids, bacterial paste and rumen of cattle). The phage isolated from these samples are specific only for *Methylosinus sporium* strains, *Methylosinus trichosporium*, and *Flavobacterium gasotypicum*. Two bacteriophages isolated from 67 fish (Tyukitov *et al.*, 1983) were found to be specific for *Methylocystis* species and *Flavobacterium gasotypicum*. Bacteriophages for other species of methanotroph have not, to date, been isolated. None of the phages so far isolated have been tested for transduction ability.

In 1984, a number of environmental samples (pond water, field run off water, soil, lake water and cattle faecal samples) were screened for methanotroph bacteriophage (Cardy and Salmond, unpublished). No phage were isolated from any of the samples tested when plated onto lawns of 14 different methanotroph strains, from the University of Warwick culture collection (see Materials and Methods Section). Each of these 14 methanotroph species was also tested for the presence of temperate phages by lysogen induction studies using U.V. irradiation and mitomycin C treatment. No temperate phage were isolated in these studies.

1:5:7 Recent advances in methanotroph molecular genetics.

The DNA encoding the γ subunit of protein A of the methane monooxygenase (MMO) complex has been cloned from the obligate methanotroph *M. capsulatus* (Bath) (Mullens and Dalton, 1987). This DNA was obtained by screening a *M. capsulatus* *Sau*3A DNA library in pDR720 with synthetic oligonucleotide probes (17-mers) corresponding to the N-terminal amino acid sequence of the γ subunit of protein A. A recombinant clone (pLM001) was isolated from the gene library, which was found to contain the γ gene by hybridization analysis and subsequent DNA sequencing of the 5' end of the gene.

The DNA encoding the α, β and γ subunits of MMO protein A of *M. capsulatus* (Bath) has subsequently been cloned and sequenced (Murrell *et al.*, 1988. Manuscript in preparation). The isolation and sequencing of the α, β and γ genes of protein A provides the first step in a molecular biological analysis of the MMO gene complex.

The cloning of the methanol dehydrogenase gene (*mxaF*) from *M. capsulatus* (Bath) and *Methylomonas albus* (BG8) has recently been reported (Stephens *et al.*, 1988). Genetic analysis of functions necessary for methanol oxidation (MOX functions) in the facultative serine pathway, methanol utilizer, *Methylobacterium* sp. strain AM1 have been previously reported (Nunn and Lidstrom, 1986(a, b)) and in *Methylobacterium organophilum* XX (Machlin *et al.*, 1988). From these studies, it appeared that the MOX system was quite complex in the facultative serine pathway, methanol utilizers, but it was not known whether it was similarly complex in the obligate methanotrophs. Stephens *et al.* (1988) utilized *Methylobacterium* sp. strain AM1 as an alternative host system for addressing questions of methanotrophic MOX functions. Putative *mxaF* genes were isolated from *M. capsulatus* (Bath) and *M. albus* BG8 by heterologous hybridization using a portion of the *M. AM1* gene (*mxaF*) encoding a portion of the methanol dehydrogenase (MDH) structural protein, as a probe. The identity of the two cloned genes was then confirmed in two ways:- i) A T7 expression vector was used to produce MDH protein in *E. coli* from the cloned genes - identification of the encoded protein was by way of immunoblotting with antiserum against the *M. albus* MDH; ii) A *mxaF* mutant of *M. AM1* was complemented to a methanol-positive phenotype, using broad host range plasmids containing the *mxaF* genes from each methanotroph.

The results presented by Stephens and his colleagues show that it is possible to obtain information concerning MOX genes from obligate methanotrophs by using mutants and gene probes from facultative methylotrophs. They also suggest that a similar approach should be useful

for studying other MOX functions from methanotrophs as well as transcriptional regulation of MDH in the obligate methanotrophs, by the use of broad host range promoter probe vehicles.

Work recently published by Oakley and Murrell (1988) has shown the presence of *nifH* (encoding the Fe protein component of nitrogenase) homologues in all but one strain of obligate methanotrophs which had previously been shown capable of fixing dinitrogen (Murrell and Dalton, 1983(a)). Interestingly, three of the six Type I non-diazotrophic methanotroph strains also showed homology to the *Klebsiella pneumoniae* *nifH* probe, and the degree of homology was found to be variable discriminating between three *Methylomonas methanica* strains, with strain A4 showing the strongest homology and strain FH exhibiting no detectable homology at all. The third Type I methanotroph showing a very weak homology to *nifH* was *Methylobacter capsulatus* Y. Oakley and Murrell postulated that the presence of these *nifH* homologues among the Type I organisms was perhaps indicative of a loss of dinitrogen fixing ability by a Type I progenitor strain, from which the resulting degree of sequence diversification, without selection, varied from strain to strain.

From this initial work, the structural genes for nitrogenase (*nifHDK*) genes have been isolated from *M. capsulatus* (Bath) and were found to be contiguous and in the order *nifH*, *nifD*, and *nifK*. These cloned *nif* genes have been shown to encode polypeptides of sizes that are consistent with those expected for these three *nif* gene products (Oakley and Murrell, manuscript in preparation).

The paucity of data concerning molecular biology and genetics of obligate methanotrophs, is largely due to the lack of both mutants and high frequency genetic transfer systems. However, the new tools afforded this area by recombinant DNA techniques and wide host range conjugative plasmids are beginning to prove successful.

1:6 Nitrogen assimilation in microorganisms

In all biological systems, nitrogen is an essential component for growth. Inorganic nitrogen compounds such as dinitrogen, nitrate, nitrite or ammonia (often the preferred nitrogen source), as well as complex organic compounds, such as the amino acids arginine or histidine, may serve as nitrogen sources for the growth of microorganisms. All nitrogen sources, whether simple or complex, can be converted enzymatically to ammonia before true assimilation of the nitrogen can occur. The exact array of usable nitrogen compounds is a characteristic of each organism.

There are a number of excellent recent reviews on nitrogen metabolism in microorganisms, including those of; Magasanik, 1982, Merrick, 1982, Kustu *et al.*, 1986 and Merrick, 1988(a)). Therefore, this section will briefly outline the various pathways of ammonia assimilation which are found in microorganisms and Section 1:7 will outline the regulation of the genes involved in nitrogen metabolism. This section will concentrate mainly on work carried out on the Enterobacteriaceae, namely *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella aerogenes* and *Klebsiella pneumoniae*, for which a complete description of nitrogen assimilation has been achieved. Information obtained from work on other bacterial genera will also be included, to highlight the similarities and differences on the modes of regulation of nitrogen assimilation in microorganisms.

1:6:1 Pathways of ammonia assimilation in microorganisms

In most microorganisms, ammonia is assimilated into cell biomass via the intermediates glutamine and glutamate. These intermediates play a central role in nitrogen metabolism, the amide group of glutamine, serving as the direct nitrogen donor in the biosynthesis of certain amino acids, purines, pyrimidines and other nitrogenous compounds. Glutamate serves as

the primary amino group donor for practically all amino acids.

(a) The role of glutamine synthetase (GS).

In all microorganisms, glutamine can only be synthesised by the action of the enzyme, glutamine synthetase (GS). GS catalyses the formation of glutamine by the addition of ammonia to glutamate, in an ATP-dependent reaction.

The most extensive studies of GS have been carried out on *E. coli* by Stadtman, Ginsburg, Holzer and colleagues (reviewed in Ginsburg and Stadtman, 1973). They found that when the extracellular concentration of ammonia in the growth medium was high, GS rapidly lost its ability to form glutamine, whereas, upon removal of excess ammonia, biosynthetic activity of GS was restored. GS from enteric bacteria, is a dodecameric protein of M_r 600,000 consisting of 12 identical subunits each of M_r 50,000. Stadtman and his colleagues (Stadtman *et al.*, 1970), showed that in *E. coli*, a rapid loss in biosynthetic activity occurs when an adenylyl group is attached to a specific tyrosine residue on each subunit. This adenylylation was found to be reversible with maximum biosynthetic activity of GS being obtained when the enzyme is completely unadenylylated.

GS activity is regulated at three different levels:-

i) Adenylylation/deadenylylation control.

Adenylylation of GS is carried out by a specific enzyme, adenylyl

transferase (ATase) in an ATP dependent reaction. ATase can also act in reverse to deadenylylate GS, its function in turn being controlled by a regulatory protein P_{II}. The form of the P_{II} protein determines whether GS is adenylylated or deadenylylated. Two forms of the P_{II} protein exist, unmodified P_{II} which stimulates ATase to adenylylate GS, and a modified form of P_{II} in which uridylyl groups are attached, which stimulates ATase to deadenylylate GS. Uridylylation of the P_{II} protein is accomplished by the enzyme uridylyl transferase (UTase), which also possesses the uridylyl removing activity (UR).

Intracellular levels of glutamine and α ketoglutarate ultimately regulate GS activity. A high glutamine: α ketoglutarate ratio, stimulates the UR activity of UTase to deuridylylate P_{II}, which in turn stimulates the adenylylation of GS by ATase. When the glutamine : α ketoglutarate ratio is low, the reverse sequence occurs (see Fig. 1:2) (Ginsburg and Stadtman, 1973).

As GS from *E. coli* consists of 12 sub-units, the enzyme can therefore exist in different states of adenylylation, ranging from the non-adenylylated GS (E_0), which has maximum biosynthetic activity, to the fully adenylylated form, GS (E_{12}), which is biosynthetically inactive (see Stadtman *et al.*, 1970).

The X-ray crystal structure of GS from *S. typhimurium* has recently been determined (Almassy *et al.*, 1986) (see Fig. 1:3). The positioning of residue 397 (tyrosine), the site of adenylylation, was determined. It was found to be at the end of an extended, exposed segment of polypeptide at the outer surface of the molecule. An unusual aspect of the active site was observed in that it was formed by two polypeptide chains. One surface of the GS active site was found to be formed mainly of 6 β-strands of the C-domain of one subunit, and the other surface by two β-strands of the N-domain of the adjacent subunit. The formation of an active site from the intimate association of two subunits, although unusual, is not unique to

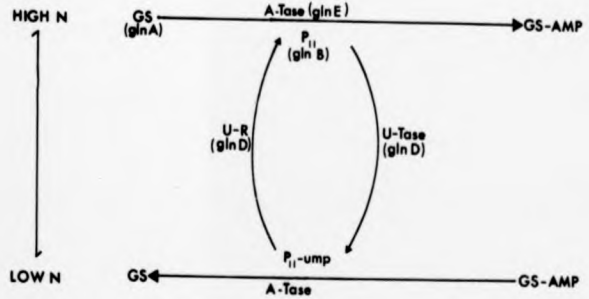

HIGH N
GS
(glnA)
A-Tase (glnE)
GS-AMP
P_{II}
(glnB)
U-R
(glnD)
U-Tase
(glnD)
P_{II}-ump
LOW N
GS
A-Tase
GS-AMP

Figure 1:2 Regulation of glutamine synthetase activity by adenylylation.

Taken from Merrick, (1988(a)).

Key:

GS - deadenylylated glutamine synthetase (active).

GS-AMP - adenylylated glutamine synthetase (inactive).

A-Tase - adenylyltransferase.

U-Tase - uridylyltransferase.

U-R - uridylyl-removing enzyme.

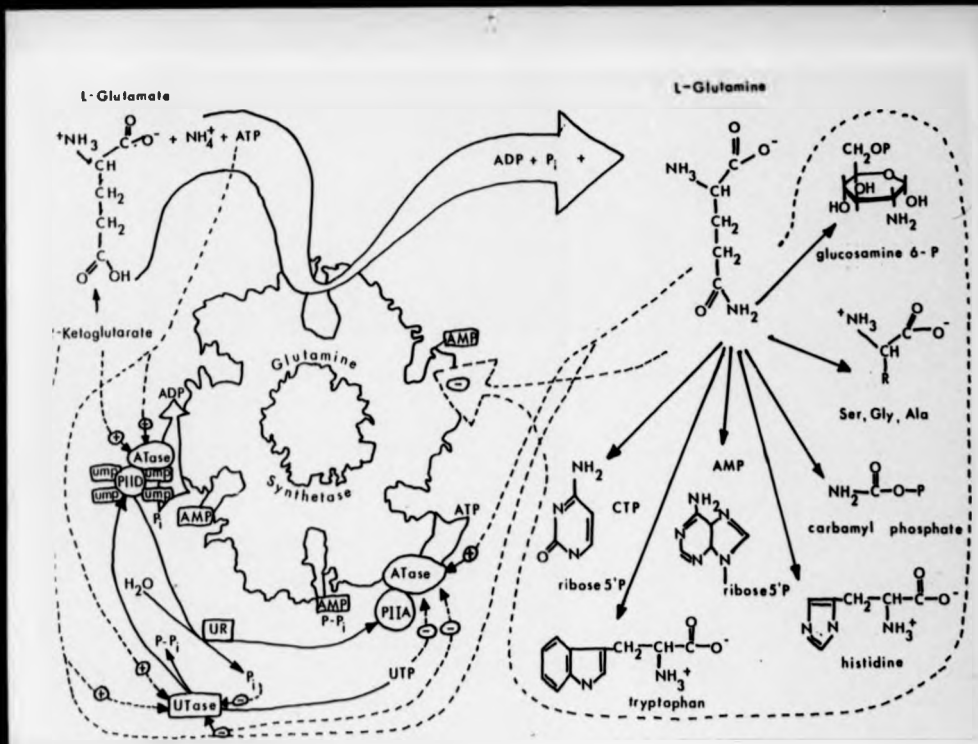

L-Glutamate
L-Glutamine
ADP + Pi +
α-Ketoglutarate
Glutamine
Synthetase
ATase
PII
UMP
AMP
ADP
ATP
PIIA
UR
UTase
UTP
H2O
P-Pi
glucosamine 6-P
Ser, Gly, Ala
CTP
ribose 5'P
carbamyl phosphate
histidine
tryptophan

Figure 1:3 Interactions of glutamine synthetase.

The ATP-dependant condensation of ammonia with glutamate is shown catalyzed at one active site of the GS molecule. The amide nitrogen of glutamine is a source of metabolites shown to the right. Each of these end products is a feedback inhibitor of GS acting at separate sites. Feedback inhibition is represented by the arrow with a negative sign. GS subunits are covalently modified by adenylylation through a phosphodiester linkage to a specific tyrosyl hydroxyl, Tyr 397 in the sequence of GS from *S. typhimurium*. Adenylylation is catalyzed by the enzyme adenylyltransferase (ATase) aided by a tetrameric regulatory protein P_{II}. This reaction is shown at the lower right of the GS molecule, where the ATase-P_{II}A complex converts ATP and an unadenylylated subunit site into an adenylylated subunit and pyrophosphate. Deadenylylation is shown on the left side of the GS molecule. Removal of the adenylyl groups is catalyzed by the same two proteins, but for removal P_{II} is modified by uridylylation. Uridylylation of P_{II} is catalyzed by transferase (UTase), shown at the bottom left of the figure. Hydrolytic removal of the UMP groups from P_{II} is catalyzed by the uridylyl removing enzyme (UR). These three levels of enzymatic catalysis in the formation of glutamine respond sensitively to concentrations of metabolites.

Taken from Almassy *et al.*, (1986).

glutamine synthetase. This type of structure has also been identified in studies of aspartate amino transferase (Ford *et al.*, 1980) and glutathione reductase (Thieme *et al.*, 1981).

The structural genes encoding GS (*glnA*) and P_{II} (*glnB*) have been identified and cloned from a number of organisms (see Table 1:3 for details).

ii) Modification of GS by divalent cations.

Glutamine synthetase from *E. coli* can be inactivated by the removal of the divalent cations Mn^{2+} or Mg^{2+} and hence, fluctuations in the free concentrations of specific divalent cations in the cell are probably important in regulation of this enzyme. Removal of Mn^{2+} inactivates GS by causing a "relaxation" in the protein structure, which in turn, leads to an exposure of sulphydryl groups and an increased susceptibility to dissociation of subunits. Addition of divalent cations, particularly Mn^{2+} and Mg^{2+} to the "relaxed" enzyme, results in a change to the "taut" enzyme configuration and complete restoration of catalytic activity (Segal and Stadtman, 1972). A change in the metal ion specificity of GS is also influenced by adenylylation of GS. Mg^{2+} is required for biosynthetic activity of the deadenylylated form of GS, whereas the adenylylated enzyme will catalyse the biosynthetic reactions at low rate, in the presence of Mn^{2+}, but not Mg^{2+} (Kingdon *et al.*, 1967).

iii) Cumulative feedback inhibition.

Glutamine synthetase activity in *E. coli* has also been shown to be regulated through feedback inhibition, by multiple end-products of glutamine metabolism, e.g. AMP, CTP, carbomyl phosphate, tryptophan, histidine and glucosamine-6-phosphate. The effect of each of these

Table 1;3 Cloned *gln* genes.

Gene	Organism	Reference
glnA	*Escherichia coli*	Covarrubias *et al.*, 1980
		Backman *et al.*, 1981
	Klebsiella pneumoniae	de Bruijn & Ausubel, 1981
		Espin *et al.*, 1982
	Salmonella typhimurium	Koduri *et al.*, 1980
	Azotobacter vinelandii	Toukdarian & Kennedy, 1986
	Thiobacillus ferrooxidans	Barros *et al.*, 1985
	Anabaena 7120	Fisher *et al.*, 1981
	Agrobacterium tumefaciens C58	Rossbach *et al.*, 1988
	Bradyrhizobium japonicum	Carlson *et al.*, 1985
	Azorhizobium caulinodans ORS571	Pawlowski *et al.*, 1987
		(refs. within)
	Rhizobium meliloti	Somerville & Kahn, 1983
	Rhizobium leguminosarum	Filser *et al.*, 1986
	Bordetella pertussis	Brownlie *et al.*, 1986
	Methylococcus capsulatus (Bath)	This Work.
	Vibrio alginolyticus	Maharaj *et al.*, 1986
	Azospirillum brasilense	Borzouklian & Elmerich, 1986
	Bacillus subtilis	Fischer *et al.*, 1984
	Clostridium acetobutylicum	Usdin *et al.*, 1986
	Streptomyces coelicolor	Rawlings - Pers. Comm.
glnB	*Escherichia coli*	Stauffer *et al.*, 1981
	Rhizobium leguminosarum	Colonna-Romano *et al.*, 1987
	Klebsiella pneumoniae	Holtel & Merrick, 1988.

inhibitors on GS activity is cumulative. This phenomenon has also been observed with glutamine synthetases isolated from other organisms outside of the Enterobacteriaceae; for example, *Azotobacter vinelandii* (Kleinschmidt and Kleiner, 1978) and *Rhodopseudomonas capsulatus* (Johansson and Gest, 1976). In organisms such as *Bacillus stearothermophilus* and *Anabaena* CA, for which there is no evidence for regulation of GS activity by adenylylation, cumulative feedback inhibition is thought to be the main mechanism by which GS activity is regulated (Wedler *et al.*, 1976; Stacy *et al.*, 1979).

GS is not only regulated at the level of activity but is also regulated at the level of expression in the Enterobacteriaceae and a number of other organisms. The system which mediates this control is known as the nitrogen regulation or *ntr* system. This will be described in detail in Section 1:7.

In the Enterobacteriaceae, glutamate, unlike glutamine, can be produced by three main types of reaction:-

i) from ammonia and α ketoglutarate, either by a reaction catalysed by glutamate dehydrogenase (GDH) (L-glutamate: $NADP^+$ oxidoreductase) or as a result of a coupled reaction catalysed by GS and glutamate synthase (GOGAT) (glutamine (amide): 2-oxoglutarate amino transferase oxidoreductase) (see Figure 1:4).

ii) as a direct degradation product of another amino acid.

iii) by a transamination reaction utilizing amino groups of other amino acids and α ketoglutarate.

b) The role of glutamate dehydrogenase (GDH).

Glutamate dehydrogenase catalyses the reductive amination of a ketoglutarate by ammonia in a reversible reaction utilizing either NADPH or NADH. GDH's are known to occur in a wide variety of bacteria (Tyler, 1978). In microorganisms, NAD-dependant GDHs appear to serve a catabolic function, whilst the enzymes utilizing NADPH, serve primarily for the biosynthesis of glutamate. Most bacteria possess only one type of GDH activity, either NADH, or NADPH dependant. However, some bacteria show two distinct GDH activities, for example, in *Hydrogenomonas* H16 (Kramer, 1970). The highest content of NADPH-linked enzyme was found in cells growing with an excess of ammonia, whereas the NADH-linked enzyme was found in high concentrations only when ammonia was growth limiting, suggesting that this enzyme was acting catabolically. Kramer suggested that only the NADPH-linked enzyme had a predominantly biosynthetic function in bacteria. In enteric bacteria only the NADPH-linked GDH is present, and is a hexamer of identical subunit M_r of 50,000 (Sakamoto *et al.*, 1975). GDHs appear to be of significance in ammonia assimilation, only when the extracellular concentrations are very high, due to their high Km values for ammonia and a ketoglutarate (of the order of 1 mM for both), which suggests that for cells growing under conditions of ammonia limitation, this enzyme is relatively ineffective in nitrogen assimilation. GDH activity in both *K. aerogenes* and *K. pneumoniae* is repressed when cells are grown with a limiting source of nitrogen (Maers *et al.* 1970; Shanmugam *et al.*, 1975). However, in *Escherichia coli* and *Salmonella typhimurium*, this repression does not occur (Brenchley *et al.*, 1975; Streicher *et al.*, 1976). The difference in the regulation of GDH in these organisms is not at present understood, however, work carried out on *K. aerogenes* has thrown some light as to the genetic mechanism underlying GDH regulation in this organism (see Section 1:7).

Figure 1.4 Possible pathways of ammonia assimilation in microorganisms

The structural genes encoding GDH have been cloned from *S. typhimurium* (Miller and Brenchley, 1984) and *E. coli*, from which the gene has been completely sequenced (McPherson and Wootton, 1983; Valle *et al.*, 1984). The deduced amino acid sequence from the *E. coli gdh*A sequence has shown a high degree of homology to the *Neurospora crassa* enzyme and hybridization analysis with the cloned *S. typhimurium gdh*A gene has also shown significant homology to sequences in the *Saccharomyces cerevisiae* chromosome, which suggests a strong conservation between corresponding enzymes in prokaryotes and eukaryotes (in Merrick, 1988(a)).

c) The role of glutamate synthase (GOGAT).

Prior to 1970, it was generally accepted that microorganisms assimilated ammonia primarily via GDH with aspartate ammonia lyase, leucine and valine dehydrogenases being of secondary importance in ammonia assimilation. Then in 1970, a major discovery was made by Meers and his colleagues of the enzyme glutamate synthase (glutamine (amide): 2-oxoglutarate amino transferase oxido-reductase) or GOGAT (Tempest *et al.*, 1970; Meers *et al.*, 1970). These workers observed that, during growth in chemostat culture of *Aerobacter aerogenes* under ammonia limiting conditions, the cells were essentially devoid of GDH and that the organism was assimilating ammonia in a two step process involving GS and GOGAT. These reactions involved the initial assimilation of endogenous glutamate to glutamine in an ATP requiring reaction catalysed by GS, followed by a reductive transfer of the amide nitrogen of glutamine to the 2-position of 2-oxoglutarate (a ketoglutarate), catalysed by GOGAT. This resulted in the net synthesis of 1 mole of glutamate from one mole of ammonia (Fig. 1:4). Subsequent observations on mutants of *K. aerogenes* and *S. typhimurium* (Brenchley and Magasanik, 1974; Rosenfeld *et al.*, 1982) lacking GDH activity and yet having no discernible phenotype (i.e. glutamate

independant), support the existence of this second pathway of glutamate biosynthesis.

Since the discovery of GOGAT, this enzyme has been found to exist in all prokaryotes subsequently studied. In some organisms such as *Azotobacter vinelandii* (Kleiner, 1975) and Type II obligate methanotrophs (Murrell and Dalton, 1983(b)) for example, ammonia is assimilated exclusively by the GS/GOGAT pathway.

Glutamate synthase has been purified from *E. coli* (Miller and Stadtman, 1972; Mantsala and Zalkin, 1976(a, b); Geary and Meister, 1977) and from *K. aerogenes* (Geary and Meister, 1977; Trotta *et al.*, 1974). The enzyme from both sources is an iron-sulphur flavoprotein, which exists as a dimer of unequal subunit structure. The smaller of the two polypeptides are essentially of the same M_r (53,000) from both organisms. The M_rs of the large subunit however, differ significantly between the two organisms; the large subunit polypeptide from *K. aerogenes* has an M_r of 175,000 whereas that of *E. coli* is lighter, with an M_r of 135,000. The composition of the GOGAT subunits *in vivo* is not clear. In *E. coli*, it has been reported that the enzyme is multimeric containing four dimers (Miller and Stadtman, 1972), whereas in *K. aerogenes*, Trotta *et al.*, (1974), have reported full GOGAT activity from a single dimer.

The structural genes for GOGAT, (*gltB* locus) are located at 69 min on the *E. coli* map, close to *argG* (Fuchs *et al.*, 1982; Garciarrubio *et al.*, 1983). As GOGAT consists of two non-identical subunits, it was expected that the ammonia assimilation negative (Asm^-) phenotype (Asm^- - mutations in GOGAT conferring inability of mutants to grow on low concentrations of ammonia, or a variety of other nitrogen sources (Brenchley *et al.*, 1973; Fahel *et al.*, 1978)), would be due to mutations in two different structural genes or possibly from an altered regulatory protein/s. Subsequent studies on a number of independent Asm^- mutants of *E. coli* (Fahel *et al*, 1978), *K. aerogenes* (Gaillardin and Magasanik, 1978) and *K. pneumoniae* (Tyler, 1978)

have shown that the loss of GOGAT activity is due to lesions tightly linked to *argG* (Pahel *et al.*, 1978). Unlike *E. coli.*, *K. aerogenes* and *K. pneumoniae*, *argG* is not co-transducible with *gltB* in *S. typhimurium*, although these two loci have been shown to lie in the same area of the chromosome (Fuchs *et al.*, 1982).

The two subunit genes of glutamate synthase have recently been cloned (Garciarrubio *et al.*, 1983; Lozoya *et al.*, 1983) and sequenced from *E. coli* (Buccaril *et al.*, 1987). The structural genes for the large (*gltB*) and small (*gltD*) subunits appear to lie in a single operon in which *gltB* appears to be transcribed first. Translational coupling of the two genes is apparent as the termination codon of *gltB* overlaps the ribosome binding site of *gltD*.

Recently, Castano and her colleagues reported the existence of a third gene, (*gltF*), downstream of *gltBD* in *E. coli*. These genes appear to lie in a single *gltBDF* operon (Castano *et al.*, 1988). The *gltF* gene codes for a polypeptide of M_r 30,200, which appears to be involved in the regulation of the *glnA ntrBC* operon. The nature of this regulation, at present, is unknown and work is in progress to determine the role of *gltF* in the regulation of nitrogen metabolism in *E. coli*.

The studies carried out on GOGAT in a number of enteric organisms revealed the regulation of this enzyme to be complex, as there is no simple correlation between levels of the enzyme with the nitrogen status of the cell (Meers *et al.*, 1970; Nagatani *et al.*, 1971; Brenchley *et al.*, 1975).

Although GS, GOGAT and GDH are the three primary nitrogen assimilation enzymes of the cell, there are however, a large number of enzymes which are concerned with the utilization of different nitrogen sources (i.e. inorganic nitrogen sources such as dinitrogen, nitrate, nitrite and organic nitrogen sources such as amino acids and specific amino acid and ammonia transport systems). The expression of some of these enzymes is regulated according to the available nitrogen source. For further details on these

enzymes and their regulation, the reader is referred to the excellent recent reviews by Stewart, 1988; Cole, 1988; Dixon, 1988 and Merrick, 1988(a, b). Some reference to these enzymes and the nature of their regulation will be made in Section 1:7 and subsequent chapters of this thesis.

1:7 Genetic control of nitrogen assimilation in prokaryotes.

In virtually all cells, glutamine and glutamate serve as the key cellular intermediates in the subsequent biosynthesis of nitrogen containing compounds. Hence, the bacterial cell must be able to synthesize the proteins which provide it with these amino acids, both under conditions of nitrogen sufficiency and also under nitrogen limiting conditions. Therefore, an understanding at the genetic level of both the structural genes which encode the enzymes involved in nitrogen metabolism, and of the genes and their products which co-ordinate and regulate the levels of these enzymes in the cell is required. Regulation of the genes involved in nitrogen metabolism has been extensively studied in the Enterobacteriaceae. This section will therefore briefly outline the state of the art mainly with respect to the Enterobacteriaceae, but will also include information on other bacterial genera for comparison.

1:7:1 Historical perspective.

In Enterobacteriaceae, the system which regulates the synthesis of proteins involved in nitrogen metabolism is known as the nitrogen regulation or *ntr* system. Research over the past decade on the regulation of the glutamine synthetase structural gene (*glnA*) (see reviews - Magasanik, 1982; Merrick, 1982; Kustu *et al.*, 1986; Merrick, 1988(a)) and also regulation of genes involved in nitrogen fixation (*nif*) (reviewed in

Dixon, 1988) has led to an understanding of this complex system. Although *nif* regulation is not directly relevant to the work carried out for this thesis, many of the components involved in *nif* regulation, are also involved in regulation of *glnA*, its associated genes, and other nitrogen-regulated systems (discussed in detail below). Therefore, some reference may be made to *nif* regulation in this section, but the reader is referred to a number of excellent, recent reviews on this subject for further details (Hasselkorn, 1986; Gussin *et al.*, 1986; Merrick, 1988(b) and Dixon, 1988).

In 1974, Magasanik and his colleagues put forward a model from studies carried out on the regulation of histidase synthesis in *K. aerogenes*, suggesting that the deadenylylated form of glutamine synthetase was the principal regulator of nitrogen assimilation. Evidence for this model included the isolation of mutants, which were believed to be in *glnA*, which showed altered regulatory properties (Magasanik *et al.*, 1974). Subsequent work by Magasanik, Tyler and their colleagues (reviewed in Magasanik, 1976; Tyler, 1978) proposed that GS acted as a genetic regulatory element, controlling transcription of its own structural gene (*glnA*), and playing a major role in the control of other genes whose products are subject to nitrogen control. Doubt as to the role of GS in regulation of nitrogen assimilation came with the isolation of Mu and Tn10 insertion mutations in the *glnA* region of *E. coli* (Pahel and Tyler, 1979) and *S. typhimurium* (Kustu *et al.*, 1979). Two classes of mutants were isolated, one class having the Gln$^-$ phenotype (inability to produce GS, resulting in glutamine auxotrophy) and the other class having the GlnR phenotype (low to intermediate concentration of glutamine synthetase, little response to alteration of nitrogen source in the medium). These mutations were identified as being in separate cistrons, *glnA* and *glnG* (*glnR*) respectively by complementation analysis. This result suggested that the product of the *glnG* gene, and not GS itself, to be the regulator of *glnA* expression. This

work, together with additional evidence against a regulatory role of GS, including: *glnA-lacZ* fusion studies in *E. coli* (Rothstein *et al.*, 1980), the subsequent identification of two cistrons *ntrB* and *ntrC* at the *glnR* locus in *E. coli* and *S. typhimurium* (the products of which proposed to be regulatory proteins for nitrogen assimilation operons) (McFarland *et al.*, 1981)) and the identification of a *glnA*-linked regulatory gene in *K. pneumoniae* (Espin *et al.*, 1980, 1981; Leonardo and Goldberg, 1980; de Bruijn and Ausubel, 1981), enabled Merrick to propose a very different model for the regulation of nitrogen assimilation (Merrick, 1982). In this model, GS was no longer considered to be a regulatory protein as the identification of three regulatory genes and their products were found to be able to exert both positive and negative control of nitrogen regulated promoters. Two of these genes, *ntrB* (alternatively, designated *glnL*) and *ntrC* (alternatively designated *glnG*), are located adjacent to *glnA* in a complex operon (McFarland *et al.*, 1981; Fahal *et al.*, 1982; Espin *et al.*, 1982; Rothman *et al.*, 1982). The close linkage of these regulatory genes with *glnA* had confounded the earlier analysis of mutants. Magasanik and Rothstein (1980), proposed that expression of *glnL* and *glnG* in *E. coli* could occur, either from its own weak promoter (intergenic between *glnA* and *glnL*), or by stronger read- through transcription from the *glnA* promoter. This model proposed that the products of *ntrB* and *ntrC* could repress or activate genes under nitrogen control, including *glnA*, and that repression required both the products of *ntrB* and *ntrC* (it was suggested that these products possibly acted as a complex). Activation, on the other hand, required only the *ntrC* and *ntrA* (alternatively designated *glnF* or *rpoN*) products. The *ntrC* product was proposed to be a DNA binding protein, with the product of *ntrB* being either a DNA binding protein or a modifier of the *ntrC* product. The product of the *ntrA* gene was thought to be a positive regulatory factor, required for the formation of a functional *ntrC* activator.

In the proceeding 6 years, since the publication of the model for nitrogen control in enteric bacteria by Merrick, rapid advances in genetic analysis of nitrogen regulation in the Enterobacteriaceae have been made, enabling the *ntr* system to be extensively characterized.

The following sub-section will therefore briefly describe our current knowledge of the *ntr* system in the Enterobacteriaceae, presenting an up to date model for *ntr* control of *glnA* and associated genes together with the implications of this model, as applied to other nitrogen-regulated systems.

1;7;2 Regulation of nitrogen assimilation in the Enterobacteriaceae.

1;7;2;1 The *ntr* system in the Enterobacteriaceae.

The genes encoding the three primary regulatory proteins, NtrA (alternatively designated RpoN σ^{54} or *gpntrA*), NtrB (alternatively designated NR_{II} or *gpntrB*) and NtrC (alternatively designated NR_I or *gpntrC*) have been cloned (in some cases sequenced), and the gene products purified from *E. coli*, *S. typhimurium* and *K. pneumoniae*. This, in part, has enabled the determination of the molecular mechanism of nitrogen regulation in the enteric bacteria.

a) *ntrA*.

This is a positive regulatory gene first described by Garcia *et al.* (1977) who stated the requirement for the product of this gene for the synthesis of GS in *S. typhimurium*. The *ntrA* gene is unlinked to *glnA ntrBC* and maps near *argG* (Garcia *et al.*, 1977; Pahel and Tyler, 1979; Leonardo and Goldberg, 1980). The transcriptional organisation of *ntrA* is not known but the expression of *ntrA* has been demonstrated by the use of gene fusions (*ntrA-tet* (de Bruijn and Ausubel, 1983); and *ntrA-lacZ* fusions (Castano and

Bastarrachea, 1984; Merrick and Stewart, 1985) to be transcribed constitutively at a low level, independant of nitrogen status of the cell. The *ntrA* gene products of *S. typhimurium* (Hirschman *et al.*, 1985), *E. coli* (Magasanik, 1982) and *K. pneumoniae* (de Bruijn and Ausubel, 1983; Merrick and Stewart, 1985) have been identified as a protein of M_r 73,000, 75,000, 84,000 and 76,000 respectively on SDS PAGE. However, subsequent sequencing of the *ntrA* gene from *K. pneumoniae* has revealed NtrA to be a protein of M_r 53,926 and the aberrant mobility of this protein on SDS-PAGE has been attributed to its acidic nature (pI < 5.0) (Merrick and Gibbins, 1985). Mutations in *ntrA* cause a variety of phenotypes including Gln^-, Ntr^- and Nif^- phenotypes. The positive regulatory role played by NtrA requires the products of the *ntrC* or *nifA* genes for initiation of transcription at *ntr*-activatable promoters e.g. *glnA* or *nif* (Garcia *et al.*, 1977; Kustu *et al*, 1979; de Bruijn and Ausubel, 1981, 1983; Pahel *et al.*, 1982; MacNeil *et al.*, 1982; Merrick, 1983).

The function of NtrA became apparent after certain findings were reported:-

i) DNA sequence analysis of eight *nif* promoters (Beynon *et al.*, 1983; Drummond *et al.*, 1983) established a consensus *nif*-promoter sequence (CTGGYAYR-N_4-TTGC) extending from -26 to -10. The sequences of these promoters differ to the canonical -35 and -10 consensus, which typifies prokaryotic promoters recognized by RNA polymerase holoenzyme containing σ^{70}.

ii) Sequencing of promoters subject to positive control by the *ntr* system i.e. those subject to *ntrC*-mediated activation, like the enteric *glnA* promoters (Dixon, 1984; Reitzer and Magasanik, 1985) and the *argT*r promoter from *S. typhimurium* (Ames and Nikaido, 1985) contained a similar, but not identical consensus to the *nif* promoters, with a consensus GG-N_{10}-GC motif

between -24 and -12 (Dixon, 1984). The GG and GC dinucleotides at -24 and -12 respectively are separated by one turn of the DNA helix and are invariant. The absolute conservation of the nucleotides in the -24 and -12 region was demonstrated by deletion analysis through the construction of five deletions between the -12 and -24 elements in the *nif* H promoter. The deletion of a single non-conserved nucleotide eliminated transcriptional activation (scored by relief of multicopy inhibition of chromosomal *nif* expression) (Buck, 1986). This work suggested that the spacing between the two dinucleotides is critical and that deletion of bases in this spacer region may disrupt a protein-DNA interaction which involves the simultaneous recognition of bases around the -12 and -24 promoter elements.

The importance of the invariant nucleotides at -12 and -24 has been demonstrated by site-directed mutagenesis studies of the *nifH* and *nifL* promoters (reviewed by Dixon, 1988).

These findings, together with the observation that transcription from these promoters was dependent on *NtrA in vivo* (Merrick, 1983; Ow and Ausubel, 1983), suggested that NtrA could have a role in modifying the promoter specificity of RNA polymerase (de Bruijn and Ausubel, 1983; Reynon *et al.*, 1983). *In vitro* analysis has subsequently confirmed this concept. Purified NtrA from *E. coli* has been shown to bind RNA polymerase core enzyme, and this complex is required to activate transcription from the activatable *glnA* promoter, *glnAp2* (Hunt and Magasanik, 1985). Partially purified NtrA from *S. typhimurium* in the presence of RNA polymerase core enzyme and a mutant form of NtrC allowed transcription from *glnAp2*, however, the NtrA requirement could not be substituted by purified RNA polymerase holoenzyme containing σ^{70} (Hirschman *et al.*, 1985). DNAse 1 digestion protection studies with partially purified NtrA and RNA polymerase core enzyme protected a region -4 to -40 of the *glnAp2* promoter from digestion (Kustu *et al.*, 1986). This *in vitro* data together with prior *in vivo* observations suggested strongly the identification of NtrA as

an alternative sigma factor which recognizes *ntr* and *nif* specific promoters, replacing σ^{70}. Due to the identification of NtrA as a new sigma factor, Hunt and Magasanik, (1985) have subsequently proposed that ntrA should be renamed rpoN.

The structural gene for NtrA (RpoN) has been cloned and sequenced from *K. pneumoniae* (Merrick and Gibbins, 1985), *S. typhimurium* (In: Merrick, 1988(a)), *Azotobacter winelandii* (Merrick *et al.*, 1987(a)) and *Rhizobium meliloti* (Ronson *et al.*, 1987(a)). Although the predicted NtrA polypeptides are highly homologous, comparative amino acid sequence analysis of the *K. pneumoniae* NtrA polypeptide and other sigma factors does not indicate it as being a member of the family of sigma factors e.g. RpoD, HtpR, SpoIIG, SpoIIAC and SigB. However, all these sigma factors were found to have two conserved sequences at their C-terminal end, resembling sequences found in known site-specific DNA-binding domains. A similar pair of sequences were identified at the C-terminus of NtrA, possibly involved in recognition of *ntr* activatable promoters (Merrick and Gibbins, 1985). All NtrA sequences available to date have been shown to possess a potential DNA-binding domain near the C-terminus, the role of which remains to be elucidated. In *K. pneumoniae*, *A. winelandii* and *R. meliloti* a highly conserved open reading frame (ORF) encoding an 11 Kd protein has been located downstream of *ntrA*. In *K. pneumoniae*, mutations in this region increase the apparent activity of NtrA *in vivo*. The precise role of the ORF and its mode of action are to date unknown (In: Merrick, 1988(a)). The role of NtrA in positive regulation of *ntr* activatable promoters will be discussed in detail later.

b) ntrB

As stated earlier, *ntrB* together with *ntrC* form part of a complex operon with *glnA* in the Enterobacteriaceae. The *ntrBC* genes lie downstream

of *glnA* and can be expressed either by transcription from one or two promoters upstream of *glnA*, or from an intercistronic promoter between *glnA* and *ntrB*. The expression of *ntrBC* under nitrogen limiting conditions is primarily from the downstream, positively regulated, *glnA* promoter (*glnAp2*), whilst under conditions of nitrogen excess, *ntrBC* expression is primarily from the intercistronic promoter initiating at *pntrBC*. The expression of these genes has largely been elucidated using *lacZ* fusions (Pahel *et al.*, 1982; Krajewska-Grynkiewicz and Kustu, 1984; Alvarez-Morales *et al.*, 1984) or S1 nuclease mapping (Ueno-Nishio *et al.*, 1984; MacFarlane and Merrick, 1985). DNA sequence analysis, transcript mapping data and β-galactosidase fusions have determined that the majority of transcripts (up to 80% from β-galactosidase fusion studies - Alvarez-Morales *et al.*, 1984; Pahel *et al.*, 1982) terminate at a *rho*-independent terminator sequence in the *glnA-ntrBC* intergenic region (MacFarlane and Merrick, 1985).

Up until 1986 the precise function of the *ntrB* product was unclear. A variety of phenotypes were observed on mutation of *ntrB*, including high constitutive synthesis of GS (GlnC), low constitutive synthesis of GS (GlnR) and supression of *glnD*, *glnB* and *ntrA* mutations to allow growth without exogenous glutamine (Magasanik, 1982; MacNeil *et al.*, 1982; Chen *et al.*, 1982). These variety of phenotypes eventually led Backman *et al.* (1983) to propose that NtrB was able to modulate the repressor or activator functions of NtrC in response to signals (perhaps from the *glnD* and *glnB* products) concerning the nitrogen status of the cell. Prior to this, NtrB had been suggested to form a complex with NtrC under certain conditions (McFarland *et al.*, 1981; MacNeil *et al.*, 1982; Wei and Kustu, 1981).

Genetic analysis of *ntrB* has been hampered in the past due to a number of factors:-

i) definitive mapping of *ntrB* mutations has been made difficult due to the relative positions of *ntrB* and *ntrC* on the chromosome.

ii) the *ntrBC* genes are cotranscribed and consequently mutations in *ntrB* can be polar on *ntrC*. This polarity has made the phenotypic characterization of *ntrB* mutants and complementation analysis with *ntrB* and *ntrC* mutations difficult.

Recent genetic studies by MacFarlane and Merrick (1987) together with biochemical studies on NtrB by Ninfa and Magasanik (1986) have led to the elucidiation of the role of NtrB in nitrogen regulation. The work by Ninfa and Magasanik (1986) using purified NtrB from *E. coli*, demonstrated that, in the presence of ATP, NtrB was shown to catalyse the transfer of the γ phosphate of ATP to NtrC. This covalently modified NtrC was shown to be active in promoting transcription initiation at *ntr*-activatable promoters.

The work of Ninfa and Magasanik has recently been supported by recent work on *S. typhimurium* NtrB and NtrC by Kaener and Kustu (1988). They demonstrated rigorously that NtrB is a protein kinase and that it could phosphorylate itself, whereas NtrC could not. Also NtrC-P was shown to be capable of autodephosphorylation as well as regulated dephosphorylation in the presence of P_{II}, NtrB and ATP. Kaener and Kustu also demonstrated that a purified amino-terminal fragment of NtrC (approximately 12.5 KDa) was sufficient for recognition by NtrB and is also capable of autodephosphorylation and regulated dephosphorylation (requiring P_{II}, NtrB and ATP), hence this N-terminal fragment contained all the determinants necessary for recognition by NtrB as well as the target for phosphorylation (Kaener and Kustu, 1988).

Further work has shown that the activity of NtrB also requires the products of two additional genes, namely *glnB* and *glnD* (Buano *et al.*, 1985; Ninfa and Magasanik, 1986) (see Section 1:7:2:2, on positive and negative regulation by *ntr* genes).

In vitro manipulation of the *ntrB* gene enabled MacFarlane and Merrick (1987) to construct a number of defined *ntrB* mutations. *In vivo* analysis

of the phenotypes of these *ntrB* mutations revealed that the covalent modification of NtrC, catalysed by NtrB, affects the negative (repressor) functions of NtrC as well as the positive control functions. Also, the phenotype of the *ntrB* 'null' mutations suggested that other cellular proteins may substitute partially for NtrB in regulating NtrC activity. For example, mutations in *ntrB* which result in the constitutive activation of *glnAp2* and *pnifLA* (comparable mutations in *E. coli* leading to the constitutive synthesis of GS and histidase (Chen *et al.*, 1982) and to the constitutive phosphorylation of NtrC *in vitro* (Ninfa and Magasanik, 1986)) were produced. These mutations were found to also result in the constitutive repression of *glnApl* and *pntrBC* which suggested that the phosphorylated form of NtrC stimulates its binding to recognition sequences on the DNA in the promoter regions. This has now been established by *in vitro* experiments with purified proteins for *pnifLA* (Minchin *et al.*, 1988) and for *E. coli* *pglnA* (Ninfa *et al.*, 1987). NtrC-P in *E. coli* has been shown to catalyse the rate of open complex formation at *glnAp2* (Ninfa *et al.*, 1987).

The structural gene for *ntrB* has been sequenced from *K. pneumoniae* (MacFarlane and Merrick, 1985) and *E. coli* (Miranda-Rios *et al.*, 1987). The deduced M_r for these proteins are 38,409 and 38,647 respectively, which are in close agreement with the estimate on SDS-PAGE of M_r 36,000 (McFarland *et al.*, 1981). The native NtrB protein from *E. coli* has been identified as a dimer (Ninfa *et al.*, 1986).

c) *ntrC*

The *ntrC* gene product (NtrC) has been purified from *E. coli* (Reitzer and Magasanik, 1983), *S. typhimurium* (Hirschman *et al.*, 1985) and *K. pneumoniae* (Hawkes *et al.*, 1985). The structural gene for *ntrC* has been sequenced from *E. coli* (Miranda-Rios *et al.*, 1987) and *K. pneumoniae*

(Buikema *et al.*, 1985; Drummond *et al.*, 1986). The derived polypeptides from these sequences have M_rs of 52,205, 52,259 and 52,340 respectively, which agrees well with the observed M_rs of 50,000 and 53,000 respectively on SDS-PAGE (Reitzer and Magasanik, 1983; Merrick, 1983; Hawkes *et al.*, 1985). The NtrC protein behaves as a dimer on purification and binds to specific sites on DNA *in vitro* (Reitzer and Magasanik, 1983; Ames and Nikaido, 1985; Hawkes *et al.*, 1985). NtrC is a DNA binding protein which has a helix-turn-helix motif at its C-terminus in common with other DNA binding proteins (see later) (Hirschman *et al.*, 1985; Drummond *et al.*, 1986). Genetic studies have shown that the *ntrC* gene product is a bifunctional regulatory protein which can act positively to activate transcription of a number of nitrogen-regulated operons e.g. in *K. pneumoniae nifLA* and *glnA* genes (Drummond *et al.*, 1983; Merrick, 1983; Ow and Ausubel, 1983) as well as acting negatively to repress transcription from p*ntrBC* and *glnA*p1 (Alvarez-Morales *et al.*, 1984; Dixon, 1984). Activator and repressor functions of NtrC are modulated by the *ntrB* gene product *in vivo* (Alvarez-Morales *et al.*, 1984; Dixon, 1984; MacFarlane and Merrick, 1987). For NtrC to function as an activator it requires the product of the *ntrA* gene (*rpoN*) (de Bruijn and Ausubel, 1983; Merrick and Stewart, 1985). A range of phenotypes are observed on mutation of *ntrC*, ranging from glutamine auxotrophy to unregulated low levels of *glnA* expression, together with an Ntr$^-$ phenotype (Wei and Kustu, 1981; MacFarland *et al.*, 1981). DNase and dimethylsulphate protection experiments, in the *glnA* promoter regulatory region of *S. typhimurium*, revealed the presence of five closely spaced binding sites for NtrC binding within 110 base pairs of the major transcriptional start site (Hirschman *et al.*, 1985). Kustu and her colleagues reported the major features of these binding sites:-

i) Each binding site has two-fold rotational symmetry.

ii) Each half-site has the consensus sequence 5' - GGTGC - 3' (or 5'- GCACC - 3'). This is in agreement with the consensus dyad symmetrical sequence GCAC-N_7-GTGC, which is typical of known DNA-binding sites for regulatory proteins (Dixon, 1984).

iii) Five binding sites are present; sites 1, 2 and 5 have a 10 base pair spacing between the half sites, equivalent to one turn of the helix (i.e. the NtrC dimer would make contacts on one side of the DNA helix).

iv) Sites 3 and 4, the distance between the half-sites is only 7 base pairs.

v) By alteration of NtrC concentration in DNase I protection experiments, NtrC was found to have the highest apparent affinity for the binding sites furthest upstream (1 and 2), lower affinity for sites 3 and 4 and the lowest affinity for site 5 (which is closest to the major start point of transcription) (Kustu *et al.*, 1986).

The significance of this large number of NtrC binding sites in the *glnA* promoter-regulatory region of *S. typhimurium* is not known. Subsequent *in vitro* experiments with purified proteins for the *E. coli glnA* promoter region however, showed that the active form of NtrC (NtrC-P or NR1-P) did not increase the ability of NtrA containing RNA polymerase to bind to the promoter, but rather it stimulated the conversion of an initial promoter:polymerase complex to the transcriptionally active open complex. The high affinity NtrC-P binding sites were shown to be sufficient for the facilitation of transcription, whereas sites 3, 4 and 5 appeared to play no

role in the activation of open complex formation in the transcription system used. The presence of sites 1 and 2 resulted in a 4-5 fold lowering of the concentration of NtrC required for formation of the open complex. It was proposed that the role of the high affinity binding sites for NtrC may be to increase the local concentration of NtrC-P at the promoter, favouring the interaction of NtrC-P with RNA polymerase-NtrA which results in the formation of the open complex. However, these high affinity NtrC binding sites still facilitate open complex formation when they are moved to positions 700 bp further upstream or 950 bp downstream of *glnAp2* on linear templates (Minfa *et al.*, 1987). This work was in agreement with results obtained by Reitzer and Magasanik (1986), who found that if these sites were relocated as much as 1400 bp further upstream, they still had a significant effect on transcriptional activation. The precise role of NtrC in activation of transcription still remains to be elucidated. The ability of bound NtrC-P to activate transcription at a distance from the transcription initiation site may involve the formation of a DNA loop to bring the bound RNA polymerase - NtrA and NtrC-P into close proximity (see Ptashne, 1986), hence allowing co-operative interaction between bound proteins and/or RNA polymerase.

Work recently reported by Buck and co-workers lends support to the DNA loop theory and appears to closely parallel the situation found in *glnA*. This work involved the increase of spacing between the upstream activator sequence (UAS) (the NifA binding site) and the -24, -12 NtrA dependent sequence in the *nifH* promoter region. Increases of full and half turns of the DNA helix revealed a significant decrease in transcriptional activation. Introduction of full helical turns (1 and 2 full turns) decreased NifA-mediated activation of the *nifH* promoter to a lesser extent than did the introduction of the half turns. The insertion of the *lac* operator within intervening sequence, when placed *in vivo*, transcriptional activation from the *nifH* promoter was not influenced by the presence of the

lac repressor. This result indicated that the bound protein (*lac* repressor) to the *lac* operator in the intervening DNA sequence, did not inhibit the interaction i.e. by the prevention of the activator sliding between binding sites. These results suggest that interaction is brought about by the formation of a DNA loop between upstream and downstream promoter elements (Buck *et al.*, 1987(a)). NifA and NtrC are functionally homologous proteins i.e. transcriptional activators, and the data described above is consistent with the DNA looping model occurring at these promoters (reviewed in Dixon, 1988; Merrick, 1988(b)).

Subsequent analysis of nucleotide sequence and derived amino acid sequence of NtrB/NtrC has revealed an unexpected degree of conservation amongst a family of regulatory proteins. These proteins belong to two component regulatory systems. One component of each system is thought to act as an environmental sensor (sensory component), which transmits a signal to the second component (regulatory component), which in turn effects the response usually to the transcriptional apparatus. Genetic studies have identified a number of these two component regulatory systems, for example; *E. coli* responding to nitrogen limitation *ntrB/ntrC*; phosphate limitation *phoR/phoB*, osmolarity *envZ/ompR*; toxic compounds *cpxA/sfrA*; *Agrobacterium tumefaciens* genes controlling virulence in response to plant exudate *virA/virG*; and C_4-dicarboxylate transport activating genes in *Rhizobium leguminosarum*, *dctB/dctD*.

NtrB belongs to the sensory class of proteins in which the C-terminal 200 amino acids are conserved. NtrC belongs to the regulator class of proteins in which the N-terminal 120 amino acids are conserved, and in some cases a greater degree of homology is identified. A model for signal transduction has been proposed due to this conservation which provides an insight into the functions of the various domains of the sensor and regulator proteins (Nixon *et al.*, 1986; Reviewed in Ronson *et al.*, 1987(b)). The model states that the N-terminal domain of the sensor

component perceives an environmental signal, possibly by the binding of a ligand if the sensor contains periplasmic domains, and transmits a signal to the conserved cytoplasmic domain via an allosteric alteration (N.B. All members of the sensory class with the exception of NtrB have hydropathy profiles consistent with an N-terminal transmembrane structure, which in turn is consistent with the known functions of these proteins, i.e. respond to external stimuli. NtrB on the other hand, responds to an intracellular signal, i.e. the nitrogen status of the cell). The C-terminal portion of the sensor protein is thus activated, interacts with and modifies the conserved N-terminal portion of the regulator protein. The modified N-terminal domain is then able to effect the response through an interaction, causing a switch in the conformation of the C-terminus between, inactive and active or repressor and activator forms.

The genetics of these two component systems have been extensively studied, in particular; *ntrB/ntrC*, *envz/ompR* and *phoR/phoB* and in most cases, as predicted by the above model, mutations in genes of either partner cause a variety of regulatory defects, ranging from noninducibility to constitutive or aberrant expression. Results supporting the contention that the C-terminal domain of the sensory component physically interacts with the N-terminal domain of the regulatory component, comes from work carried out on the *envZ/ompR* system by Matsuyama *et al.* (1986). They found that second-site suppressors of *envZ11* (*envZ11* mutation modifies Thr247 in the conserved C-terminal domain) mapped within the conserved N-terminal region of ompR (modified Leu16 in this region). This suppressor mutation produced no apparent phenotype in a wild-type background.

The regulator class of proteins also shares homology at their N-termini with the products of the sporulation regulatory genes, *spo0A* and *spo0F*, of *Bacillus subtilis* and the chemotaxis signal processing genes, *cheB* and *cheY*, from enteric bacteria (Ronson *et al.*, 1987(b) and references therein). These gene products have not been shown to function in

association with a component homologous to the sensor class.

NtrC has three major domains within its predicted polypeptide sequence. Its N-terminal 120 residues shows significant homology to the family of regulatory proteins mentioned above. This is followed by a short linker sequence of approximately 20 residues, very hydrophilic, and is predicted to be predominantly coil or turn, proposed to be a relatively mobile linker on the surface of the molecule. Insertion of 4-8 codons in this linker region of NtrC (or NifA - see later) does not affect either the degree of activation or the kinetics of the regulatory response. This shows that the linker has no role in transmitting conformational shifts from the 'regulatory' N-terminal domain to the rest of the molecule (Drummond, 1988). The central domain consists of approximately 240 amino acids and shows homology to a comparable region in NtrC of *Bradyrhizobium parasponiae* and *Rhizobium meliloti*, in NifA of *K. pneumoniae*, *R. meliloti* and *R. leguminosarum* and in DctD of *R. meliloti* (Nixon *et al.*, 1986; Drummond *et al.*, 1986; Szeto *et al.*, 1987; Ronson *et al.*, 1987(b)). The domain at the C-terminus is comprised of approximately 65 amino acids and contains a potential DNA binding motif, capable of forming a helix-turn-helix structure, homologous to that found in many DNA binding proteins. This potential DNA binding motif is found in the C-terminal domains of NtrC, NifA and DctD (Drummond *et al.*, 1986; Merrick, 1987).

The products of the regulatory operons *nifLA* (regulates expression of nitrogen fixation genes) and *ntrBC* (regulates a number of nitrogen regulated operons including *nifLA*) have a number of similarities. The NifA and NtrC proteins show considerable functional similarity, in that, both require the *ntrA* product (NtrA) for activation. These proteins are not only functionally homologous proteins but also show considerable structural homology at the amino acid level (Buikema *et al.*, 1985; Drummond *et al.*, 1986). The N-terminal domains of these proteins are not homologous, although the N-terminal domain of NtrC does show homology with the family

of regulatory proteins previously mentioned. The central domain regions of NifA and NtrC are highly conserved in both proteins, which may reflect their function in activation of *ntr* and *nif* regulated operons i.e. it may interact with NtrA containing RNA polymerase. The C-terminal domains contain a helix-turn-helix motif, characteristic of site specific DNA binding proteins (Drummond *et al.*, 1986). The recognition helices of these two proteins are not homologous, which is as expected, as the binding sites on the DNA for NifA and NtrC are different (see Dixon, 1988). The demonstration that this C- terminal domain determines the DNA-binding properties of these proteins has been achieved by both chemical and oligonucleotide-directed mutagenesis studies (Contreras and Drummond, 1988(a), (b); Morett *et al.*, 1988). p*glnA* contains high affinity NtrC binding sites, p*nifLA* contains only two weak NtrC binding sites located at -142 and -163 (Wong *et al.*, 1987). The consequence of this is, the concentration of NtrC required for activation of p*nifLA* transcription is 5-10 fold greater than that required at the *glnA* promoter (Wong *et al.*, 1987). Mutations in the weak NtrC binding sites of p*nifLA* have been suppressed by specific point mutations in the helix-turn-helix motif of NtrC, which confirms that interactions between this domain and its binding sites does occur (Contreras and Drummond, 1988 (a), (b)).

Both NifA and NtrC appear to be modified in response to fixed nitrogen by the products of the genes with which they are cotranscribed, NifL and NtrB (Ninfa and Magasanik, 1986). Genetic analysis of *ntrBC* and *in vitro* analysis of the functions of NtrB and NtrC (described previously) demonstrated that NtrB is required for the activation and inactivation of NtrC, which is achieved by phosphorylation and dephosphorylation respectively (Ninfa and Magasanik, 1986). NifA and NtrC in their conserved central domain contain a potential nucleotide binding pocket (Drummond and Wootton, 1987), which may play a role in the phosphorylation of NtrC. However, covalent modification of NifA has not been demonstrated. Arnott

et al. (1988) have constructed a number of in-frame deletions of the *K. pneumoniae nifL* gene and analysed their effects on NifA-mediated regulation of *nifH* expression, in order to determine whether NifL had an analogous role to NtrB. Previous work had determined partial homology of *K. pneumoniae* NifL with *K. pneumoniae* and *Bradyrhizobium japonicum* NtrB proteins, as well as C-terminal homology to the sensor family of regulatory proteins (Drummond and Wooton, 1987). The work of Arnott and colleagues showed that deletions of *nifL* affecting both N and C terminal domains of NifL, all gave the same phenotype, namely, loss of both nitrogen and oxygen control of *nif* expression. They concluded that unlike NtrB, NifL is only required for inactivation of its respective partner, NifA (Arnott *et al.*, 1988).

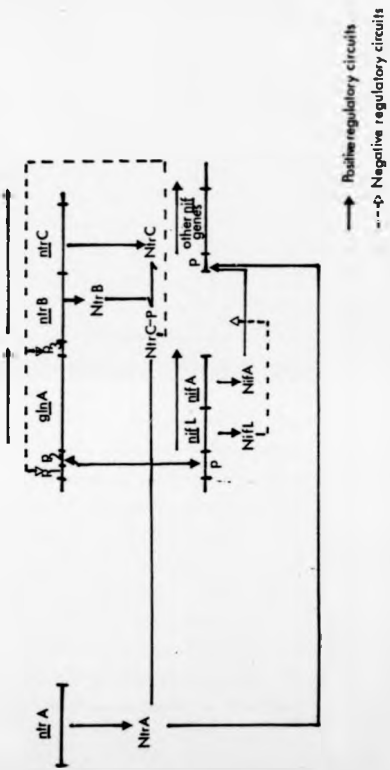
1.7.2.2 The role of the *ntr* system in positive and negative regulation of *gln A* expression.

In enteric bacteria, the *glnA ntrBC* operon contains three promoters, which are positively or negatively regulated by the products of the *ntr* genes. Two of these promoters lie upstream of *glnA* (*glnAp1* and *glnAp2*) and the other promoter being intergenic between *glnA* and *ntrB* (pntrBC). The products of the *ntr* system positively regulate *glnAp2* and also negatively regulate *glnAp1* and p*ntrBC* (see Figure 1:5).

Promoters subject to positive control by the *ntr* system such as *glnAp2*, require the presence of an activator protein, NtrC (or NifA for *nif* promoters; Merrick, 1983) and NtrA (σ^{54}) containing RNA polymerase, as well as NtrB (although work carried out by MacFarlane and Merrick (1987) on defined *ntrB* mutations, suggested that other cellular proteins may substitute partially for NtrB in regulating NtrC activity, which in turn affects both positive and negative control functions). Negatively regulated promoters are not NtrA-dependent; they have the typical -10 and

Figure 1.5 Model for the regulation of *ntr* regulated operons.

Modified from Alvarez-Morales *et al.*, (1984) and Dixon, (1988).



-35 consensus which typifies prokaryotic promoters and are expressed therefore by RNA polymerase containing the major sigma factor RpoD (or σ^{70}), negative control being exerted by the action of NtrB and NtrC proteins.

Hence, under conditions of nitrogen limitation, NtrB catalyses the covalent modification, by phosphorylation, of NtrC. The phosphorylated form of NtrC is active in promoting transcription initiation from *ntr*-activatable promoters i.e. *glnAp2* (Minfa and Magasanik, 1986) and due to its improved DNA-binding properties, repressing transcription from p*ntrBC* and *glnAp1* (MacFarlane and Merrick, 1987). Repression is obtained at the negatively controlled promoters during nitrogen limiting conditions, due to the presence of NtrC binding sites flanking the transcription initiation site and when occupied by NtrC-P, impedes the binding of RNA polymerase. For example, in p*ntrBC*, a single NtrC-binding site flanks the transcription initiation sites so that on binding of NtrC-P, RNA polymerase binding is impeded and expression from p*ntrBC* is prevented (Dixon, 1984; MacFarlane and Merrick, 1985). Using an *in vitro* coupled transcription-translation system, Hawkes *et al.* (1985) demonstrated the binding of purified NtrC from *K. pneumoniae*, to *glnAp1* and p*ntrBC*. Mutagenesis studies on *ntrB* (MacFarlane and Merrick, 1987), indicated the effect of mutant NtrB protein on NtrC activity. Mutations in *ntrB* which led to constitutive phosphorylation of NtrC caused constitutive expression from *ntr*-activatable promoters, and strong repression at p*ntrBC* (which is independent of nitrogen status). Hence, when NtrC is phosphorylated (during nitrogen limiting conditions), expression from *ntr* activatable promoters is initiated and repression at p*ntrBC* and *glnAp1* is maximal. In nitrogen excess conditions, dephosphorylation of NtrC occurs, which leads to a loss of *glnAp2* expression, and the repression from *glnAp1* and p*ntrBC* relieved. NtrC-P inhibition of expression is achieved by the presence of strong NtrC binding sites flanking the transcription initiation site (only one strong

NtrC binding site is present in *K. pneumoniae* whereas *E. coli* and *S. typhimurium* have two), such that during nitrogen limiting conditions, NtrC-P binding inhibits expression from *glnApl* (Dixon, 1984; Reitzer and Magasanik, 1985).

The phosphorylation state of NtrC determines the functional state of *ntr*-activatable promoters. The phosphorylation state of NtrC is mediated by NtrB, the phosphatasing or kinase functions of which, are determined by the nitrogen status of the cell. The modulation of NtrB activity is regulated by the cascade system which regulates GS at the level of activity (adenylylation/deadenylylation cascade). The product of the *glnB* gene, the P_{II} protein, modulates NtrB activity. Under conditions of nitrogen excess, the product of the *glnD* gene (uridyl-removing enzyme) deuridylylates P_{II}. *In vitro* studies have shown that this form of P_{II} interacts with NtrB to promote dephosphorylation of NtrC. Under conditions of nitrogen limitation, P_{II} becomes uridylylated by the *glnD* product (uridylylating enzyme), this form of P_{II} cannot interact with NtrB and hence NtrC is phosphorylated (Ninfa and Magasanik, 1986) (see Figure 1:6).

Recently the *glnB* gene of *K. pneumoniae* has been cloned and the wildtype, as well as two mutant alleles of *glnB*, sequenced (Holtel and Merrick, 1988). The *glnB* gene from *E. coli* has also been recently sequenced (Son and Rhee, 1987) and showed almost complete homology to the *K. pneumoniae glnB* gene. The uridylylation site of P_{II} has been identified as Tyr51 and analysis of a point mutation of *glnB* (*glnB502*) was found to change Glu50 to Lys50, yielding a Gln^- phenotype. This Gln^- phenotype is thought to be due to the above mutation preventing uridylylation of Tyr51. This mutant was also found to be Nif^- and failed to activate *nifLA*, maybe due to the inability of NtrB to escape P_{II} control and hence unable to phosphorylate NtrC. A Tn5 insertion within 20 bp of the original *glnB502* mutation suppressed this mutation to Gln^+. This Tn5 insertion would prevent P_{II} synthesis. Strangely, this mutant showed almost wild-type

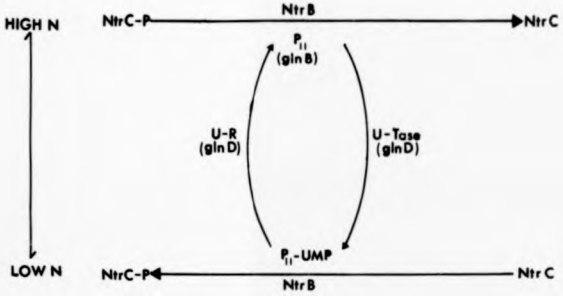

HIGH N
LOW N
NtrC-P
NtrB
NtrC
P_II
(glnB)
U-R
(glnD)
U-Tase
(glnD)
P_II-UMP
NtrC-P
NtrB
NtrC

Figure 1:6 Regulation of covalent modification of NtrC protein.

Taken from Merrick, (1988(a)).

Key:

NtrC-P - phosphorylated NtrC protein - active in positive control of *ntr* activatable promoters.

NtrC - dephosphorylated NtrC - inactive in positive control.

nitrogen control of *nifA* expression. The absence of P_{II} did not appear to affect the ability of the *ntrBC* system to respond to the nitrogen status of the cell (Holtel and Merrick, 1988). The ability of the *ntr* system to regulate the synthesis of a number of proteins in response to the nitrogen status of the cell, centres around the ability of the *glnD* product (GlnD) to uridylylate/deuridylylate the *glnB* gene product, P_{II}. The activity of GlnD is in turn determined by the intracellular ratios of α ketoglutarate to glutamine.

1;7;2;3 Co-ordination of nitrogen assimilation in the Enterobacteriaceae.

The ability of enteric bacteria to co-ordinate the activities of a large number of potential pathways for nitrogen assimilation, under any particular growth condition, is achieved in most by the *ntr* system. As stated previously, GDH, GOGAT and GS are the three principal enzymes of nitrogen metabolism, and, of these, only the expression of GS is markedly regulated in response to nitrogen status. This regulation of GS is achieved both at the level of expression and activity, which in turn are determined by the intracellular ratio of α ketoglutarate to glutamine. GS therefore, ultimately regulates its own synthesis and activity. The function of the *ntr* system in governing expression of other nitrogen assimilation systems, is also regulated by the intracellular ratio of α ketoglutarate to glutamine. Hence, the state of the glutamine pool and the activity of GS is central to the whole nitrogen economy of the cell. The evolution therefore, of several levels of GS regulation has enabled the system to respond rapidly and precisely to changes in nitrogen status.

GDH and GOGAT, the other principal enzymes of nitrogen metabolism, appear to be subject to far less control in response to nitrogen status and less appears to be known about the nature of their regulation. Studies carried out by Bender and his colleagues on *K. aerogenes* (*glnA5*, *glt*B200)

double mutants (resulting in glutamate auxotrophy), alluded to the existence of a gene designatd *nac* (nitrogen assimilation control). These double mutants were glutamate auxotrophs due to the *glnL45* mutation, resulting in the GlnC phenotype (constitutive *glnA* expression) plus severe repression of glutamate dehydrogenase and the *gltB200* mutation resulting in elimination of glutamate synthase. Study of revertants to glutamate prototrophy revealed one class of revertant which showed high levels of GDH and an Ntr$^-$ phenotype i.e. unable to use histidine or proline as sole nitrogen-source. These mutants mapped in a gene designated *nac*, which was found to be linked to the histidine (*his*) operon on the *K. aerogenes* chromosome. Studies of a *nac* mutant (*nac*-1) revealed that under nitrogen limiting conditions, the expression of GDH and GOGAT was not reduced. These results suggested that the *nac* gene encoded a repressor protein responsible for the repression of GDH and GOGAT. Subsequent work on the *nac* gene of *K. aerogenes* has been carried out by Macaluso and Bender (1986). This work revealed, by use of MudAp*lac* fusions to the *nac* gene, the requirement for *ntr*A and *ntr*C gene products for expression. This suggested that *nac* itself was under positive *ntr* control. The fact that *nac* mutants cannot utilize poor nitrogen sources such as histidine or proline suggest that, direct control of *hut* or *put* occurs via *nac* and only indirectly by *ntr*.

The role of *nac* in regulation of other nitrogen control systems remains to be alluded. It has been proposed that *nac* could possibly be involved in the regulation of systems which can provide the cell with carbon as well as nitrogen (Merrick, 1988(a)). An understanding of the mechanism of *nac* control awaits its molecular characterization and also elucidation of the genes it regulates.

Other systems for which there is good evidence for direct *ntr* control are the amino acid transport systems. For instance, the glutamine permease operon (*glnHPQ*), encoding the high-affinity transport system of glutamine

in *E. coli* has been cloned and sequenced (Mohno *et al.*, 1986). Analysis of the promoter region revealed the presence of two canonical *E. coli* promoter sequences, *glnHp1* and *glnHp2*. The more downstream of the two promoters, *glnHp2*, showed strong homology to NtrA dependent promoters (Mohno and Saito, 1987). On subsequent analysis of this promoter region (not reported), it can be noted that 100 bp upstream from *glnHp2* is a sequence showing strong homology to the consensus NtrC binding site. This evidence suggests that the *glnHPQ* operon may be subject to *ntr* regulation. There is also good evidence that the transport systems for histidine, arginine, glycine and ornithine are under direct *ntr* control. NtrA consensus sequences have been found in the promoter regions of the *hisJQMP* (*dhuA* region) and *argT* (*argTr* region) of *S. typhimurium*, and by the use of Mud 1 (Ap, *lac*, *cts*) fusions, NtrC and NtrA were demonstrated to be required for transcription activation (Stern *et al.*, 1984; Ames and Nikaido, 1985). Further indication that these genes were under *ntr* control came from work carried out by Kustu and colleagues who showed that, when grown on a poor nitrogen source, elevated levels of the respective proteins were noted (Kustu *et al.*, 1979). Subsequent studies on the *argTr* and *dhuA* regions of *S. typhimurium*, when fused to the galactokinase (*galK*) gene, showed *argTr* to be under nitrogen control. However, the *dhuA* region did not appear to be regulated by nitrogen (Schmitz *et al.*, 1987). This unexpected result was thought to be due to a mechanism of regulation at *dhuA*, occurring via anti-termination of transcripts initiating in the upstream operon, within *argTr*. Hence, under these conditions, increased expression of the chromosomal histidine transport operon would occur via transcriptional read-through. Supporting this hypothesis, the NtrC binding site in *dhuA* was found to overlap a *rho*-independent termination site for nitrogen-regulated transcripts initiating upstream. Preliminary data appears to indicate anti-termination occurring within *dhuA* (in Schmitz *et al.*, 1987).

A few amino acid degradative pathways have also been characterized genetically in the Enterobacteriaceae. The degradative pathways for histidine (*hut*), arginine (*aut*), proline (*put*) and tryptophan (*tut*) have been elucidated. A number of genes encoding various enzymes of these degradative pathways appear to be under nitrogen control. However, differences exist between different organisms within the Enterobacteriaceae and the link between *ntr* and regulation of amino acid degradation is in the early stages of analysis. For an up-to-date review on this area of nitrogen metabolism the reader is referred to the review by Merrick, 1988(a).

1:7:3 Regulation of nitrogen assimilation outside the Enterobacteriaceae.

Although the physiology and biochemistry of nitrogen assimilation has been studied in a wide variety of bacterial genera, the regulation of nitrogen assimilation at the molecular/genetic level had been largely neglected in bacteria other than the Enterobacteriaceae. As our knowledge and understanding of the regulation of nitrogen assimilation at the molecular/genetic level in the Enterobacteriaceae has increased in recent years, this has stimulated analogous studies in other bacterial genera, in order that systems of nitrogen regulation in these organisms may be compared and contrasted. Even so, there is still a considerable amount of work yet to be carried out in the enterics i.e. the mechanisms controlling the expression of GOGAT and GDH are still unclear, as well as the role of the *ntr* system in regulation of amino acid degradation.

Studies carried out on a number of bacterial genera have revealed the presence of a system analogous to the *ntr* system of the enteric bacteria, as well as the presence of different mechanisms for mediating nitrogen assimilation.

1:7:3:1 Gram positive organisms.

In Gram positive organisms such as *Bacillus subtilis* and *Clostridium acetobutylicum*, there is no evidence as to the presence of a global *ntr* system. The *glnA* genes have been cloned from these organisms and expressed in the heterologous host *E. coli* (Fisher *et al.*, 1984; Schreier *et al.*, 1985 and Usdin *et al.*, 1986). The expression of the cloned *B. subtilis glnA* in *E. coli* was stimulated under nitrogen limiting conditions such that a ten-fold increase was observed, compared to that observed under nitrogen sufficient conditions. Mutations in the *E. coli ntr* genes or *glnA*, *glnB*, *glnD* or *glnE* genes had no effect on this observed regulation. *B. subtilis glnA* promoter region - *lacZ* fusion studies demonstrated that the *B. subtilis glnA* gene is negatively autoregulated i.e. the *B. subtilis glnA* gene product is necessary for regulation of its own synthesis. The mechanism by which this autoregulation is achieved is to date unknown and it has been postulated that some factor other than the *glnA* product itself, coded for by the *glnA* gene region or some cellular metabolite may act as the repressor of *glnA* expression, under conditions of nitrogen sufficiency (Schreier and Sonenshein, 1986). There is no evidence to indicate that the *B. subtilis* GS is regulated at the level of activity, either by adanylylation (Fisher and Sonenshein, 1984) (or by an *E. coli in vitro* system (Deuel *et al.*, 1970)), or by feedback inhibition by the end products of glutamine metabolism (Deuel *et al.*, 1974). Other studies on *Bacillus* spp. have also not revealed any evidence to the presence of an *ntr* system. For example, the levels of the nitrogen catabolic enzymes; histidase, arginase and alanine dehydrogenase, although dependant upon the nitrogen source present in the growth media, induction of these occurs even in the presence of preferred nitrogen sources, e.g. ammonia (Schreier *et al.*, 1982).

Studies on the cloned *glnA* gene from *C. acetobutylicum* in *E. coli* have shown expression to be regulated by levels of nitrogen from its own regulatory region (Usdin *et al.*, 1986). Subsequent studies have also determined that unlike *B. subtilis*, the region downstream of the *C. acetobutylicum glnA* structural gene is involved in the regulation of GS synthesis (Janssen *et al.*, 1988). On sequencing the *glnA* region from *C. acetobutylicum*, Janssen and his colleagues noted that this region contained three putative, extended promoter consensus sequences (P_1, P_2 and P_3), which were characteristic of Gram-positive bacteria. P_1 and P_2 were located upstream of the *glnA* gene and P_3 was located downstream and orientated towards the *glnA* gene. All three promoters were shown to have promoter activity using promoter probe vectors. Downstream of *glnA*, but upstream of P_3, an extensive stretch of inverted repeat sequences was detected. Subsequent deletion analysis of this region revealed that the levels of GS were affected if P_3 and the inverted repeat sequences were deleted, such that levels of GS were reduced approximately five-fold under nitrogen limiting conditions, but repression of GS levels in cells grown under nitrogen-excess conditions were not affected. These results indicated that the DNA sequences downstream of the *glnA* structural gene had a regulatory role, as do the downstream genes in the Enterobacteriaceae. The downstream putative promoter P_3 and the inverted repeat region appear to play a positive role in enhancing the production of GS by the *C. acetobutylicum glnA* gene. However, the way in which this is achieved is at present unknown. Janssen and his colleagues suggest that this regulation may involve transcription from P_3 (which produces an antisense RNA complementary to 43 bases at the start of the putative *glnA* mRNA).

Alternatively, the secondary structure(s) of the downstream mRNA may play a role in termination of transcription or in the stability of the mRNA (Janssen *et al.*, 1988).

There is to date, no information on any other Gram-positive organism as to the possible existence of an analogous *ntr* system as found in the Enterobacteriaceae and also a number of other Gram-negative organisms (see Sub-Section 1:8:3:2).

In contrast to the limited information obtained from Gram-positive bacteria, several Gram-negative bacteria have been shown to possess a system analogous to the *ntr* system of the Enterobacteriaceae (see Table 1:4). However, information on the role of this analogous *ntr* system in other genera is limited.

1:7:3:2 Gram-negative bacteria.

In *Anabaena*, the formation of heterocysts and the ability to fix nitrogen are under nitrogen control. There has been one report as to the existence of an analogous system in *Anabaena* to the *ntr* system in the enterics (MacKray *et al.*, 1985). However, further evidence to substantiate this claim has never been published. The *glnA* gene from *Anabaena* 7120 has been cloned (Fisher *et al.*, 1981) and sequenced (Tumer *et al.*, 1983). Transcription from the *Anabaena glnA* gene can occur from either one of two promoters. Expression of this gene in *E. coli* occurs independant of the concentration of ammonia in the growth medium and does not require the *ntrC* gene product (Fisher *et al.*, 1981). The transcription initiation sites have been determined by Northern hybridization analysis of transcripts prepared from cells grown in two different conditions; N+ (ammonia grown) and N- (dinitrogen fixing) (Tumer *et al.*, 1983). Two major transcripts were observed during growth utilizing ammonia (N+), (RNA1 and RNA2). Only RNA1 is detected from cells utilizing molecular nitrogen and the promoter sequence which acts as a start point for this transcript resembles those of the *Anabaena nif* genes. The *Anabaena glnA* '*nif* like' promoter in no way resembles, the NtrA dependent promoters or the consensus -10, -35 promoters

Table 1.4 Cloned *ntr* genes.

Gene	Organism	Reference
ntrA	*Klebsiella pneumoniae*	deBruijn & Ausubel, 1983
		Merrick & Stewart, 1985
	Escherichia coli	Castano & Bastarrachea, 1984
	Salmonella typhimurium	Hirschman *et al.*, 1985
	Azotobacter vinelandii	Toukdarian & Kennedy, 1986
	Rhizobium meliloti	Ronson *et al.*, 1987
ntrB	*Klebsiella pneumoniae*	Espin *et al.*, 1982
	Escherichia coli	Backman *et al.*, 1981
	Salmonella typhimurium	McFarland *et al.*, 1981
	Azotobacter vinelandii	Kennedy & Toukdarian, 1987
	Bradyrhizobium parasponia	Nixon *et al.*, 1986
	Rhizobium meliloti	Szeto *et al.*, 1987
	(partially cloned)	
ntrC	*Escherichia coli*	Pahel & Tyler, 1979
	Salmonella typhimurium	McFarland *et al.*, 1981
	Klebsiella pneumoniae	deBruijn & Ausubel, 1981
	Azotobacter vinelandii	Toukdarian & Kennedy, 1986
	Bradyrhizobium japonicum	Nixon *et al.*, 1986
	Agrobacterium tumefaciens	Rossbach *et al.*, 1987
	Thiobacillus ferrooxidans	Barros *et al.*, 1985
	Azorhizobium sesbaniae ORS571	Pawlowski *et al.*, 1987
	Rhizobium meliloti	Szeto *et al.*, 1987
	Rhodopseudomonas capsulata	Haselkorn, 1986
	Methylococcus capsulatus (Bath) (partial)	This Work.

and has the sequence CAAAAC and TCTAC. Tumer *et al.* (1983) suggested that
-35 -13
these *Anabaena* sequences may represent a consensus sequence which is recognized by a different species of RNA polymerase, which contains a novel sigma factor, which in turn may mediate nitrogen control in this organism. The sequence of the start point of transcription for RNA2 on the other hand, complies with the consensus typical *E. coli* promoter, with conserved sequences at -10 and -35. The cloned *Anabaena glnA* gene functions in *E. coli glnA* mutants. This expression occurs independantly of the *ntrC* product and is not regulated by ammonia. S1 nuclease protection studies revealed RNA2 to be the major transcript (i.e. originating from the *E. coli*-like promoter). In *Anabaena* itself, the *glnA* gene is expressed at a slightly higher level during nitrogen starvation conditions, than in ammonia containing medium and the resulting gene product is not subject to adenylylation (Tumer *et al.*, 1983).

Subsequent to the work carried out on *Anabaena*, a recent report by Wagner and colleagues eluded to the existence of *ntr*-like genes in the cyanobacterium *Synechococcus* 7002. *Synechococcus* 7002 DNA, cloned into a cosmid was found to complement an *E. coli glnALG* mutant for growth on ammonia and histidine (Wagner *et al.*, 1988). Further work is required to elucidate the existence of a *ntr*-like system in other cyanobacteria, similar to that found in the Enterobacteriaceae, or whether a different mechanism for co-ordinating nitrogen assimilation exists, as in *Anabaena*.

Analogues to *ntrA* and *ntrC* genes have been isolated from *Azotobacter vinelandii* by complementation of *E. coli* mutants (Toukdarian and Kennedy, 1986). The *glnA* gene was also cloned from this organism and was found to be adjacent to *ntrC* (as in the enteric bacteria), but unlinked to *ntrA*. The presence of *ntrB* was determined by hybridization with a *K. pneumoniae ntrB* probe, to lie between *glnA* and *ntrC* (Kennedy and Toukdarian, 1987). These cloned *Azotobacter vinelandii* genes were also found to complement *K. pneumoniae* mutants as well as hybridizing to *K. pneumoniae ntrA*, *ntrC* and

glnA gene probes. Subsequent construction of mutants in *ntrA* and *ntrC* in *A. vinelandii* by Tn5 mutagenesis and marker exchange, revealed the role of these genes in this organism. Both *ntrA* and *ntrC* were found to be required for induction of nitrate reductase (Santero *et al.*, 1986; Toukdarian and Kennedy, 1986). Unlike *K. pneumoniae*, *ntrC* was not required for nitrogen fixation in *A. vinelandii*, however, *ntrA* was required. Also, mutations in *ntrC* did not effect growth of *A. vinelandii* on amino acids such as histidine, proline or arginine when used as sole nitrogen source. Recently, a new regulatory gene has been identified in *A. vinelandii* and *A. chroococcum*, designated *nfrX* (Santero *et al.*, 1988). In *A. vinelandii*, three different nitrogenases have been identified; Molybdenum (Mo), Vanadium (V) and Iron (Fe) (see Bali *et al.*, 1988). NtrA is required for expression of all three nitrogenases. NifA and NfrX have been shown to be involved in expression of Mo and Fe nitrogenases and NtrC is required for expression of V nitrogenase (Bali *et al.*, 1988).

Unlike the enteric bacteria, *A. vinelandii glnA* requires neither *ntrA* nor *ntrC* function (Santero *et al.*, 1986; Toukdarian and Kennedy, 1986). The *ntrA* gene from *A. vinelandii* has been sequenced and the deduced polypeptide shows substantial homology to NtrA from *K. pneumoniae* and *Rhizobium meliloti* (Merrick *et al.*, 1987(a)).

There is also evidence of an analogous *ntr* system in *Azospirillum brasilense*. Pedrosa and Yates (1984) have described *ntrC*-like and *nifA*-like mutants in *A. brasilense*. The *ntrC* type was complemented by plasmids carrying *K. pneumoniae nifA* or *ntrC* genes. The *nifA* type was only complemented by *nifA*. These observations strongly suggested the existence of a control of nitrogen fixation in *Azospirillum*, analogous to the *ntr/nif* control described in *K. pneumoniae*. The *A. brasilense glnA* structural gene has been cloned and sequenced (Bozouklian and Elmerich, 1986), which revealed the absence of *E. coli* canonical or *ntr* type promoters. This suggested that either *glnA* escapes *ntr* control in this organism, or control

is different to the *ntr* control found in enteric bacteria. The *ntr*C mutants of *A. brasilense* fail to grow on nitrate as sole nitrogen source, but unlike similar mutants in *K. pneumoniae*, they can utilize amino acids such as, histidine, proline and arginine as sole nitrogen sources (Pedrosa and Yates, 1984).

Cloned DNA from *Bordetella pertussis* on a broad host range cosmid vector (pLAFR1), was shown to complement an *E. coli glnALG* mutant for growth on NH_4Cl, as sole nitrogen source or arginine and proline with limiting glutamine. This data, together with observations of increased histidase levels in this mutant harbouring the recombinant cosmid under nitrogen-limiting conditions, suggest the presence of analogous nitrogen regulatory genes in *B. pertussis* (Brownlie *et al.*, 1986).

Evidence for the presence of an *ntr* system in *Pseudomonas aeruginosa* comes from the study of mutants impaired in the utilization of a number of nitrogen sources. These mutants were unable to derepress the formation of GS and urease under conditions of nitrogen limitation, whereas NADP-dependant GDH became derepressed. One class of these mutants has been shown to be closely linked to *glnA*, which may define a potential *ntr*C-like gene. Another class of mutant appears to be a potential *ntr*A homologue, as these mutants fail to use nitrate and a number of other amino acids as sole nitrogen source, as well as failing to derepress GS and urease in response to nitrogen limitation (Janssen *et al.*, 1982). Extra evidence to support the presence of an *ntr*A homologue in *Pseudomonas* species comes from the identification of the NtrA-dependant consensus, in the promoter of the *Xyl*ABC operon located on the *Pseudomonas putida* TOL plasmid (Dixon, 1986). *XylA-lacZ* fusion studies, carried out in *E. coli*, demonstrated that activation of the *XylA* promoter required the *ntr*A gene product, and that heterologous regulatory proteins NtrC and NifA from *K. pneumoniae* could substitute the homologous *P. putida XylR* gene product for activation of transcription from this promoter (Dixon, 1986). A promoter sequence

homologous to the NtrA-dependent consensus has also been identified in *P. aeruginosa* (Johnson *et al.*, 1986).

The presence of *ntr*B and *ntr*C homologues have also been identified in the photosynthetic bacterium *Rhodobacter capsulatus*. However, mutations in either of these genes do not result in an Ntr$^-$ phenotype i.e. mutants with lesions in the *ntr*C-like gene (designated *nifR*1 in *R. capsulatus*), unlike enteric bacteria, can utilize arginine or proline as sole nitrogen sources. Also, unlike the enteric bacteria, this *ntr*C-like gene is not linked to *gln*A in this organism. Mutants of *R. capsulatus* which have an Ntr$^-$ phenotype have been isolated and complemented by an *E. coli* gene, which is unrelated to the known *ntr* genes (Allibert *et al.*, 1987). The role of this gene is as yet undetermined (reviewed in Vignais *et al.*, 1988). The presence of an NtrA consensus promoter in the promoter region of the *nif*HDK operon of *R. capsulatus*, suggests that the expression of *nif* genes is under the control of the global regulatory *ntr* system (Vignais *et al.*, 1988). Also, a recent report by Kranz stated the absolute requirement of three genes for the induction of the *R. capsulatus nif* system. Two of these genes, designated *nifR*1 and *nifR*2, are homologous to *E. coli ntr*C and *ntr*B genes respectively, and are adjacent to each other on the *R. capsulatus* chromosome. The third gene, designated *nifR*4, which is located downstream of the *nif*HDK operon, has been shown by sequence analysis at its carboxyl terminus, to show strong homology and functional analogy to the *R. meliloti ntr*A gene. Kranz also reported the presence of multiple *ntr*B and *ntr*C-like genes, as determined by DNA Southern hybridization analysis, in *R. capsulatus* (Kranz, 1988).

It is clear from these studies that nitrogen control in *R. capsulatus* is complex and that further work is required in order to elucidate the complexities of this system.

In the acidophilic autotroph *Thiobacillus ferrooxidans*, the presence of *gln*A-linked *ntr*BC analogues have been determined. A cloned *T.*

ferrooxidans DNA fragment which contained the *glnA* gene, enabled an *E. coli glnALG* deletion mutant to activate histidase and also utilize arginine or low levels of glutamine as sole source of nitrogen. The expression of the Ntr phenotype by this *T. ferrooxidans* DNA suggested that *T. ferrooxidans* has regulatory *ntrB* and *ntrC* genes, which are structurally linked to *glnA* (Barros *et al.*, 1985). Subsequent sequencing of the *T. ferrooxidans nifH* gene region (Pretorius *et al.*, 1987) and *glnA* gene region (Rawlings *et al.*, 1987), revealed the presence of a consensus NtrA-dependent promoter region in *nifH* but not *glnA*, and also a well-conserved consensus NtrC binding site in the *glnA* promoter region (Rawlings *et al.*, 1988 in press).

Evidence for an analogous *ntr* system has been presented for several members of the Rhizobiaceae. An analogue of *ntrC* has been identified and cloned from the *Sesbania rostrata* stem and root nodule inducing bacterium *Azorhizobium caulinodans* ORS571. NtrC from this organism does not regulate *glnA* expression and mutations in this gene lead to a severe reduction in growth on amino acids, such as arginine and histidine as sole nitrogen source, as well as leading to $Nif^{-/+}$ (5-15% wild-type), $Fix^{-/+}$ phenotypes (Pawlowski *et al.*, 1987). An additional *ntr* locus, *ntrYX* which is distinct from, but linked to *ntrC*, has also been identified on the ORS571 genome. This additional regulatory locus appears to be involved in the control of the ORS571 *nifA* promoter (reviewed in de Bruijn *et al.*, 1988).

Using the cloned *ntrC* gene of *A. caulinodans* as a DNA hybridization probe, the *ntrC* locus of *Agrobacterium tumefaciens* C58 has also been cloned. Mutation and subsequent gene replacement of the wild-type C58 *ntrC* gene revealed the requirement of *A. tumefaciens ntrC* gene for activation of GSII (see later), nitrate reductase genes and also chromosomal, but not the Ti-plasmid borne, arginine catabolism pathways (Rossbach *et al.*, 1987). Homologues to *ntrB* and *ntrC* have also been cloned and sequenced from *Bradyrhizobium parasponiae* and the deduced products exhibited extensive homology to their counterparts in *K. pneumoniae* (Nixon *et al.*, 1986). In

Rhizobium meliloti, the *ntrC* gene has also been cloned and sequenced. The deduced amino acid sequence of this gene showed 74.5% homology to the *B. parasponiae ntrC* gene product and 44.4% homology to the *K. pneumoniae ntrC* gene product. A partial fragment of the *R. meliloti ntrB* gene was also cloned and sequenced and the deduced amino acid sequence exhibited homology to the carboxyl-terminal amino acids of the *B. parasponiae* and *K. pneumoniae ntrB* gene products (Szeto *et al.*, 1987). The cloning and sequencing of an *ntrA*-like gene from *R. meliloti* has also been reported (Ronson *et al.*, 1987(a)). The deduced *R. meliloti ntrA* gene product showed 38% homology to the *K. pneumoniae ntrA* gene product. In *Rhizobium leguminosarum*, the presence of an analogous *ntr* system has been implicated by the requirement of *ntrC* for the expression of GSII (see later) of *R. leguminosarum* in *K. pneumoniae* (Filser *et al.*, 1986) and also the C_4-dicarboxylate transport gene *dctA*, has been shown to require NtrA (Ronson *et al.*, 1987(a)) for expression. Subsequent nucleotide sequencing of dctA has revealed a consensus promoter recognized by NtrA (Ronson *et al.*, (1987(c)). Also in *Bradyrhizobium japonicum*, upstream of the GSII structural gene (*glnII*), an NtrA promoter consensus sequence, as well as an NtrC binding site, have been found (Carlson *et al.*, 1987).

Studies of mutants in these *ntr*-like genes has given an insight as to the role of the *ntr* system in these organisms. For instance, *ntrA* mutants of *R. meliloti*, as in *A. vinelandii*, fail to grow on nitrate and are also Nif$^-$ (Ronson *et al.*, 1987(a); Toukdarian and Kennedy, 1986). However, *ntrC* mutants of both symbiotic *R. meliloti* (in free living *R. meliloti* NtrC is required for *nif* expression) (Szeto *et al.*, 1987) and *A. vinelandii*, are not Nif$^-$, unlike *ntrC* mutants of *A. brasilense*, *R. capsulatus* and *K. pneumoniae*, which are Nif$^-$. Hence, NtrC does not appear to play a major role in regulation of nitrogen fixation in symbiotic *R. meliloti* or *A. vinelandii* (reviewed in Gussin *et al.*, 1986). Also, *ntrC* mutants of *R. meliloti*, as well as *A. vinelandii* and *A. brasilense*, unlike *K. pneumoniae*,

can utilize amino acids such as arginine, histidine and proline as sole nitrogen source, but fail to grow on nitrate as sole nitrogen source (Szeto *et al.*, 1987; Toukdarian and Kennedy, 1986; Pedrosa and Yates, 1984).

The Rhizobiaceae are an unusual family of organisms in that, nearly all the organisms so far studied have been found to contain two distinct glutamine synthetase enzymes, GSI and GSII (Darrow and Knotts, 1977; Fuchs and Kaister, 1980). GSI resembles the GS found in the Enterobacteriaceae in its structure, heat stability and reversible modification by adenylylation. GSII however, is heat labile, consists of 8 subunits each of M_r 36,000 and is not modified by adenylylation. However, GSII is transcriptionally regulated in response to levels of available ammonia (Rossbach *et al.*, 1987). Subsequent cloning and sequencing of the structural GSII gene from *B. japonicum* has revealed significant amino acid homology with the GS of higher plants. The presence of GSII therefore, in the Rhizobiaceae, has been suggested to be the result of genetic exchange between eukaryote and prokaryote (Carlson and Chelm, 1986). The structural gene for GSI has also been cloned and sequenced from *R. leguminosarum* and the deduced amino acid sequence is extensively homologous to the prokaryotic GS (Filser *et al.*, 1986; Colonna-Romano *et al.*, 1987). The GSI structural gene has also been cloned from *R. meliloti* (Somerville and Kahn, 1983), *B. japonicum* (Carlson *et al.*, 1985), *Agrobacterium tumefaciens* (Rossbach *et al.*, 1988), and *Azorhizobium caulinodans* ORS571. However, in contrast to all other rhizobial species, *A. caulinodans* appears to lack GSII (in de Bruijn *et al.*, 1988). *A. tumefaciens* C58 and *R. phaseoli* also appear to be unusual in that they possess a third *gln* locus (designated *glnT* and GSIII respectively), which complement *E. coli glnA* mutants. The role of these genes is as yet unknown (Rossbach *et al.*, 1988; Espin *et al.*, 1988).

A recent report showed the presence of two GS's in the symbiotic *Frankia* sp. strain CpIL. *Frankia* are Gram-positive, aerobic

sporoactinomycetes and are phylogenetically distant from the Rhizobiaceae. The presence of a second GS in *Frankia* (which is similar to GSII in Rhizobiaceae) questions the origins and uniqueness of GSII genes in members of the Rhizobiaceae. The two GS enzymes from *Frankia* sp. strains CpIL were shown to be similar in their properties and regulation to their Rhizobial counterparts (Edmands *et al.*, 1987). This begs the question as to the existence of an *ntr* system in this Gram-positive organism, as GSII is regulated positively by an *ntr* system in *Rhizobiaceae*. No *ntr*-like system has been identified in any Gram-positive organism so far studied (see Sub-Section 1;8:3;1). The relative roles of both types of GS in Rhizobiaceae and Frankia in free living and in the symbiotic states remains to be elucidated.

Information regarding regulation of nitrogen metabolism in organisms other than the Enterobacteriaceae is accumulating and advances made in studies on the enteric bacteria will hopefully be paralleled by progression in studies of other systems. The resulting information can then be compared to reveal the mechanisms by which microorganisms are able to co-ordinate nitrogen assimilation.

These studies were therefore initiated to develop techniques of genetic manipulation in the obligate methanotrophs and to use ammonia assimilation as a model system due to the availability of gene probes, mutants etc., and the knowledge so far accrued. *Methylococcus capsulatus* (Bath) is the organism of choice for these studies, as the physiology and biochemistry of ammonia assimilation has been studied in detail in this organism (Murrell and Dalton, 1983(b)).

Chapter 2. Materials and Methods.

2:1 Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Tables 2:1:1, 2:1:2, 2:1:3 and 2:2 respectively.

2:2 Bacteriophage.

Bacteriophage used in this study are listed in Table 2:3.

2:1 Bacterial strains.

Table 2:1:1 Methanotrophs.

Type I	Type II
Methylomonas albus BG8	*Methylosinus sporium* 5
Methylomonas agile A20	*Methylosinus sporium* 12
Methylomonas methanica S1	*Methylosinus trichosporium* PG
Methylomonas methanica A4	*Methylosinus trichosporium* OB3b
Methylomonas methanica PM	*Methylosinus trichosporium* OB4
	Methylosinus trichosporium OB5b
Methylobacter capsulatus Y	*Methylocystis parvus* OBBP

Type X

Methylococcus capsulatus (Bath)

These organisms are all described in Whittenbury *et al.*, 1970(a) and were obtained from the University of Warwick culture collection.

Table 2:1:2 *Escherichia coli* strains.

Strain	Genotype	Source	Reference
DH1	F$^-$, *recA1*, *endA1*, *gyrA96* *thi-1*, *hsdR17* (r_K M_K), *supE44*, λ^-.	C. Oakley	Hanahan, 1983
HB101	F$^-$, *hsdS20* (r_B, M_B), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, *xyl-5*, *mtl-1*, *supE44*, λ^-	C. Oakley	Boyer and Roulland-Dussoix, 1969
CSH26ΔF6	F$^-$, *ara*, Δ(*lac-pro*), *thi*, *rpsL*, Δ(*recA-srl*)F6, *sup*^{0} λ^-.	D. Gill	Jonas & Holland, 1984
TH16	*thi*, *endA*, *hsr*, Δ*lac*U169, Hut^+_K, $hutC_K$, *glnA* :: Tn5	B. Magasanik	B. Magasanik (pers. comm.)
ET8000	*rbs*, *lacZ*::IS1, *gyrA*, $hutC_K$.	A. Toukdarian	MacNeil *et al.*, 1982
ET8894	*rbs*, *lacZ*::IS1, *gyrA*, $hutC_K$, Δ(*rha-ntrC*) 1703::Mu*cts*62	A. Toukdarian	MacNeil *et al.*, 1982
ET8556	*rbs*, *lacZ*::IS1, *gyrA*, $hutC_K$, *ntrC* 1488.	A. Toukdarian	Merrick, 1983.

ET8045	*rbs*, *lacZ*::IS1, *gyrA*, $hutC_k$, *ntrA* 208::Tn10	A. Toukdarian	MacNeil *et al.*, 1982.
TG1	(*pro-lac*)$^{\nabla}$ *supE*, *thi*, F', *traO*, *proAB*$^{+}$, *lacI*^{q}, *lacZ*$^{\nabla 15}$	C. Oakley	D. Gill pers comm.

Table 2:1:3 *Klebsiella pneumoniae* strains.

Strain	Genotype	Source	Reference
KP5022	*his*D2, *hsd*R1	M. Merrick	Streicher *et al.*, 1974
CK273	*his*D2, *ntr*A2273, *hsd*R1	M. Merrick	Toukdarian & Kennedy, 1986
UNF1848	*his*D2, Δ(*gln*A, *ntr*B,C)218 Δ*lac*Z 2003, *rec*A56, sb1300::Tn10	M. Merrick	Alvarez-Morales *et al.*, 1984.

Table 2:2 Plasmids

Plasmid	Characteristics	Phenotype	Source	Reference
pBR325	Multicopy cloning vector	Ap^r Tc^r Cm^r	C. Oakley	Bolivar, 1978.
pBR327	Multicopy cloning vector	Ap^r Tc^R	S. Evans	Soberon *et al.*, 1980.
pVK100	Cosmid vector	Tc^R Km^R	C. Oakley	Knauf & Nester, 1982.
pSP6	Cosmid vector	Sp^R Sm^R	R. Breadon	R. Breadon (pers. comm.)
pGSS33	Broad host range cloning vector	Tc^R Cm^R Ap^R Sm^R	R. Breadon	Sharpe, 1984.
pRK2013	Conjugative plasmid	Km^R ColE1 Mob^+ Tra^+	D. Hodgson	Figurski & Helsinki, 1979.
pRK2501	Broad host range cloning vector	Tc^R Km^R	D. Hodgson	Haas, 1983.
pKT231	Broad host range cloning vector	Km^R Sm^R	D. Hodgson	Bagdasarian *et al.*, 1981.

pAM51	pACYC184::*glnA*$_K$	Tc^R	M. Merrick	Alvarez-Morales *et al.*, 1984.
pAN509	pBR322::*glnA*$_A$	Ap^R Tc^R	J.C. Murrell	Fisher *et al.*, 1981.
pSM10	pBR322::*ntrB*$_K$	Ap^R	A. Toukdarian	MacFarlane & Merrick, 1987.
pMD114	pACYC177::*ntrC*$_K$	Ap^R	A. Toukdarian	Kennedy & Drummond, 1985.
pAT523	pBR325::*glnA*$^+$*ntrC*$^+_{AV}$	Ap^R Tc^R	C. Kennedy	Toukdarian & Kennedy, 1986.
pAT705	pBR325::*ntrA*$^+_{AV}$	Ap^R Tc^R	C. Kennedy	Toukdarian & Kennedy, 1986.
pMM17	pBR327::*ntrA*$_K$	Ap^R Tc^R	M. Merrick	Merrick & Stewart, 1985.
pMM26	pACYC184::*rpoD*$_E$	Cm^R	M. Merrick	Merrick & Stewart, 1985.
pAH3	pUC19::*glnB*$_R$	Ap^R	M. Merrick	Colomma-Romano *et al.*, 1987.
pAH5	pUC19::*glnB*$_E$	Ap^R	M. Merrick	Stauffer *et al.*, 1981.

pHP45Ω	pHP45::Ω	Ap^R Sm^R Sp^R	D. Gill	Prentki & Krisch, 1984.
pDC1	pBR325::$glnA_{Mc}$	Ap^R Tc^R	-	This work.
pDC2	pBR325::$glnA_{Mc}$	Ap^R Tc^R	-	This work.
pDC10	pBR325::$glnA_{Mc}$ pDC1 *Hin*dIII deletion plasmid	Ap^R	-	This work.
pDC20	pBR327::$glnA_{Mc}$ pDC100 *Hin*dIII deletion plasmid	Ap^R	-	This work.
pDC11	(1)	Ap^R	-	This work.
pDC12	(2)	Ap^R		This work.
pDC100	pBR327::$glnA_{Mc}$ (3)	Ap^R Tc^R	-	This work.
pDC110	(4)	Ap^R	-	This work.
pDC2Ω	pBR325::$glnA_{Mc}$::Ω	Tc^R Ap^R Sm^R Sp^R	-	This work.
pCoS1	pVK100::$glnA_{Mc}$ $ntrC_{Mc}$	Km^R	-	This work.

E = *Escherichia coli*

K = *Klebsiella pneumoniae*

A = *Anabaena* 7120

Av = *Azotobacter vinelandii*

R = *Rhizobium leguminosarum*

Mc = *Methylococcus capsulatus* (Bath).

(1) : pDC11 contained the 2.2 Kb *Sal*I fragment from pDC1.

(2) : pDC12 contained the 2.35 Kb *Bam*HI fragment from pDC1.

(3) : pDC100 contained the 2.7 Kb *Eco*RI-*Cla*I fragment from pDC1.

(4) : pDC110 contained the 10 Kb *Hind*III fragment from pCoS1 in the *Hind*III site of pDC20.

Table 2.3 Bacteriophages.

Phage	Characteristics	Source	Reference
λ_{ear}	b_{221} rex::Tn5 cI857 O_{am29} P_{am80}	J. C. D. Hinton	de Bruijn & Lupski, 1984.
M13tg130	-	Amersham International	Kieny *et al.*, 1983.
M13tg131	-	Amersham International	Kieny *et al.*, 1983.

2:3 Media.

2:3:1 Methanotroph media.

The basic mineral salts medium (MS) of Dalton and Whittenbury (1976) was used for routine growth of these organisms. The medium was either supplemented with 1 g litre^{-1} potassium nitrate, giving nitrate mineral salts (NMS), or with 1 g litre $^{-1}$ ammonium chloride, giving ammonium mineral salts (AMS).

For solid media, 15 g litre^{-1} of Difco bacto-agar was added to the mineral salts medium (omitting phosphates) prior to sterilization. Sterile phosphate stock solution was added aseptically to the sterile mineral salts when cool. Methane was added as carbon source as stated in Section 2:4.

2:3:2 Enterobacteriaceae media.

Rich media i.e. Luria Bertani Broth (LB), as described in Maniatis *et al.*, (1982) was used routinely to grow the enteric organisms used in this study. The medium used for the Hanahan transformation procedure (SO, SOB and SOC) have also been previously described (Hanahan, 1983).

The defined media used in these studies have also been previously described, M9 medium (Maniatis *et al.*, 1982); Nitrogen Free medium (NFDM) (Dixon, 1977); Hershey salts, H-medium and K-medium for *E. coli* 'maxi' cells as described in Worcell and Burgi (1974) and Rupp *et al.* (1971).

For solid rich media, 15 g litre^{-1} of Difco bacto-agar was added to LB media, prior to sterilization.

For solid defined media, 15 g litre^{-1} of Difco noble agar was added prior to sterilization.

2:3:3 Antibiotics.

Antibiotics, where appropriate, were used at the following concentrations (unless otherwise stated):

Antibiotics	Stock concentration mg/ml	Final concentration in media µg/ml
ampicillin	100	100
chloramphenicol	40	20
kanamycin	50	50
nalidixic acid	20	20
streptomycin	20	20
tetracycline	20	20

Antibiotic stock solutions were prepared as described in Maniatis *et al.* (1982).

2:3:4 Chemicals.

All chemicals unless otherwise stated were obtained from BDH, Sigma or Fisons and were of Analar grade. Restriction enzymes and buffers plus T4 DNA ligase, ^{35}S-methionine and ^{32}P-nucleotides were obtained from Amersham International. Organic acids and solvents were obtained from May and Baker. DNA sequencing materials e.g. acrylamide, bisacrylamide, ammonium persulphate, sodium dodecylsulphate (SDS) and urea were obtained from Bio-Rad. DNA polymerase I (Klenow) and calf intestinal alkaline phosphatase were obtained from BCL. DNA polymerase I for nick translation was obtained from BRL. Sephadex G50 was obtained from Pharmacia.

2:4 Growth and maintenance of bacterial cultures.

2:4:1 Organism maintenance.

Methanotroph cultures were maintained by sub-culturing every two weeks on NMS agar plates. The plates were then placed in 5 litre airtight plastic containers. Prior to closing the lid, methane (Anachem, U.K.) was injected into the container by means of a football bladder inflated with methane, to give a final approximate concentration of 50% v/v with air. The containers were then incubated at 45°C for *Methylococcus capsulatus* (Bath), and 30°C for all other methanotrophs.

All *Escherichia coli* strains (with the exception of TG1) and *K. pneumoniae* strains were maintained on LB agar plates containing the appropriate antibiotic and supplement where required for up to 6 weeks at 4°C. For longer term storage, an overnight LB culture was mixed with an equal volume of sterile glycerol in a glass vial and stored at -20°C.

E. coli TG1 was maintained on M9 minimal agar plates containing 0.1 ml 0.1% (w/v) thiamine litre^{-1}.

2:4:2 Growth conditions.

M. capsulatus (Bath) was grown at 45°C. Liquid cultures were routinely grown in 250 ml flasks containing either NS, NMS or AMS medium, as described and were then sealed with Suba-seal stoppers and methane injected to give an atmosphere of approximately 20% methane. Cultures were incubated on a rotary shaker at 45°C.

E. coli and *K. pneumoniae* strains were grown at 37°C unless otherwise stated in the text. Liquid cultures were routinely propagated in 25 ml universal bottles in a Gallenkamp Orbital Shaker (150 rpm).

2:4:3 Light Microscopy.

All cultures were examined by phase contrast microscopy, using an Olympus model stereoscopic microscope (X 1000 magnification). The purity of methanotroph cultures was also checked by streaking on nutrient agar plates and incubating these aerobically at 30°C and 45°C.

2:5 Chromosomal DNA extraction.

All chromosomal DNA extractions were carried out using the technique of Marmur *et al.* (1961) with modifications (Oakley and Murrell, 1988). Volumes were scaled down to allow all operations to be carried out in 35 ml polypropylene Oakridge tubes. 500 mls of chemostat grown culture (OD_{660} = approximately 6) were harvested in an MSE18 centrifuge using 6 x 250 ml rotor (6,000 rpm, 10 min, 4°C). The pellet was resuspended in 6 ml, 10 mM Tris-HCl, 1 mM Na-EDTA pH 8.0 (T.E.) and transferred to a 35 ml polypropylene Oakridge tube to which 3.75 ml 0.25 M EDTA (pH 8.0) and 50 mg lysozyme were added and mixed gently. This was placed at 37°C for 15 min. Proteinase K (250 µl of 20 mg/ml stock) was then added, followed by 3.25 ml SDS (10% w/v), which was added slowly with mixing and placed at 37°C until lysis occurred. Sodium perchlorate (4 ml of 5M) was then added (to aid DNA-membrane separation) and placed at 60°C for 15 min with occasional gentle stirring. An equal volume of (T.E. saturated) phenol/chloroform/iso-amylalcohol (25:24:1) was added and mixed gently by inversion. To facilitate complete separation of aqueous and organic phases, centrifugation in an MSE 18 centrifuge using 8 x 50 rotor (18,000 rpm, 30 min, 4°C) was carried out, and the upper aqueous layer transferred using a wide base Gilson tip to a fresh polypropylene Oakridge tube. An equal volume of chloroform was added and mixed gently by inversion, then

spun in an MSE centrifuge (18,000 rpm, 15 min, 4°C) to separate aqueous and organic phases. This extraction was repeated at least twice. After the final extraction, the aqueous phase was transferred (using a wide bore pipette) to a polycarbonate 250 ml centrifuge pot. Sodium chloride (5M) was added to a final concentration of 0.1 M, and then two volumes of 100% ethanol (stored at -20°C) was added. DNA ("wooly" in appearance) was then gently removed by spooling and washed with 70% v/v ethanol (at room temperature). The washed DNA was dried under vacuum for 30-60 min, and then slowly resuspended overnight in 10 ml TE. RNAase A (20 mg/ml in TE, heat treated; as described in Maniatis *et al.*, (1982) was added to a final concentration of 100 μg/ml, and the solution incubated at 37°C for 30 min. The chromosomal DNA was then purified by a caesium chloride (CsCl) density gradient step. The RNAse treated DNA solution was made up to 30 ml with TE into which 30 g caesium chloride was gently dissolved. Finally, 3 ml of ethidium bromide (10 mg ml^{-1}) were added and the resulting mixture placed in a 35 ml heat-sealable tube for the Beckman VTi50 rotor. Centrifugation was carried out in a Beckman L8-70 centrifuge (45,000 rpm, 16 hr, 20°C). The resulting DNA band was harvested. The ethidium bromide was removed by repeated iso-amylalcohol extractions and the caesium chloride by extensive dialysis against TE.

2;6 Isolation of plasmid DNA.

2;6;1 Large scale preparation.

The alkaline lysis method of Birnboim and Doly (1979) was used as described by Maniatis *et al.*, (1982) except solution II (alkaline-SDS solution) was not placed on ice prior to use as the SDS precipitates. Also, the inclusion of a centrifugation step (18,000 rpm, 30 min) prior to caesium chloride gradient centrifugation, removed material from the subsequent gradient.

After removal of ethidium bromide with iso-amylalcohol, plasmid DNA was directly precipitated from caesium chloride as described by Davies *et al.*, 1980.

2:6:2 Small scale preparation (mini-prep).

The alkaline lysis method of Birnboim and Doly (1979) was used as described by Maniatis *et al.* (1982). Solution II again was not placed on ice prior to use and also the 70% (v/v) ethanol wash of the DNA pellets was omitted, as this resulted in loss of plasmid.

2:7 General techniques used for DNA manipulation.

2:7:1 Extraction of DNA with phenol/chloroform/iso-amylalcohol.

Phenol/chloroform/iso-amylalcohol 25:24:1, TE saturated, was prepared and used as described by Maniatis *et al.*, 1982.

2:7:2 Precipitation of DNA.

To a DNA solution, one fiftieth volume of sodium chloride (5M) and two volumes of 100% ethanol (-20°C) were added, mixed gently and chilled at -20°C overnight. The DNA was recovered by centrifugation in an MSE Microcentaur (5 min, high speed, 4°C). All traces of the supernatant were discarded by use of a vacuum line and the DNA pellet dried under vacuum and then resuspended in TE buffer.

For DNA prepared using an I.B.I. electroeluter or during ssDNA preparation for sequencing, a half volume of ammonium acetate (7.5 M, pH 7.5) and two volumes of 100% ethanol (at room temperature) were added, mixed by vortexing and chilled at -20°C overnight and DNA recovered as described above.

2:7:3 Restriction endonuclease digestion of DNA.

Restriction endonucleases and restriction endonuclease buffers (X10) were obtained from Amersham International and used according to the manufacturers instructions, though a five-fold excess of enzyme was routinely employed to ensure complete digestion.

2:7:4 Dephosphorylation of DNA.

The terminal 5' phosphates were removed from DNA by treatment with calf intestinal phosphatase (CIP). This treatment minimized recircularization of plasmid DNA and increased the frequency of recombinant plasmid DNA molecules during ligation and transformation.

The method of D. Hodgson (pers. comm.) was used. DNA was cut with the appropriate restriction endonuclease and to every 10 μl of restriction digestion solution, 1.5 μl 0.5 M glycine pH 9.4 (NaOH); 1.5 μl $MgCl_2$ (10 mM), $ZnCl_2$ (1 mM); 1.5 μl dH_2O and 0.5 μl CIP (BCL) were added, mixed and incubated at 37°C for 30 min. The volume was then made up to 100 μl with distilled water, the DNA extracted with an equal volume of phenol/chloroform/Iso-amylalcohol, followed by a chloroform extraction and finally ethanol precipitated as described in 2:7:1 and 2:7:2 respectively.

2:7:5 Ligation of DNA.

Ligation of DNA fragments were carried out according to the recommendations of the suppliers of the enzyme (T4 DNA ligase - Amersham International), though a five-fold excess was routinely employed to ensure complete ligation. The relative concentrations of vector and insert DNA's used in the reaction were calculated in accordance with Maniatis *et al.* (1982). Ligations were carried out at 15°C for a minimum of 18 hours.

2:7:6 Agarose gel electrophoresis.

Horizontal agarose slab gels were used routinely. Slab gels were prepared by boiling Agarose (Type II medium EEO-Sigma) in 1 x Tris-borate-EDTA (TBE) electrophoresis buffer (0.89 M Tris-base, 0.89 M boric acid, 0.002 M EDTA (pH 8.0)) and cooled to *ca.* 45°C before pouring. 0.7% (w/v) gels were used routinely. DNA samples were prepared by addition of one tenth volume gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% w/v Ficoll (type 400) in dH_2O) and loaded into the gel slots. Electrophoresis was carried out in a BRL model H4 horizontal gel system with the gel completely submerged in 1 x TBE buffer at 150 volts or 60 volts when run overnight.

The DNA was stained within the agarose gels with ethidium bromide (0.5 µg/ml) as described in Maniatis *et al.* (1982) and then visualized by transillumination with short-wave UV light and photographed using Polaroid Type 665 black and white film. Restriction digests were routinely checked for completion by electrophoresis of samples on a 'mini-gel' apparatus (Cambridge Biosciences).

2:7:7 Quantitation of DNA.

The mini-gel method as described in Maniatis *et al.* (1982) was routinely used to estimate the amount of DNA in a given sample. λ (*Hin*dIII restricted) DNA was the standard DNA used (50 ng/µl) for all estimations. Restriction fragment sizes were also determined using the above standards or ϕx174 *Hae*III restricted DNA for sizing small (<500 bp) DNA fragments. Restriction mapping was performed essentially as described in Maniatis *et al.* (1982).

2:7:8 Preparation of DNA fragments from agarose gels.

DNA fragments to be eluted were separated by electrophoresis in agarose gels, 0.5% to 2% (w/v) (Section 2:7:6), depending on the size of fragment to be isolated. Once separated, the desired fragment was excised from the gel and placed in an IBI electroeluter. Fragments were electroeluted according to the manufacturers instructions. Eluted fragments were then ethanol precipitated as described in Subsection 2:7:2.

2:8 Transformation.

2:8:1 *E. coli*.

Routine transformation was carried out as described by Maniatis *et al.* (1982) with the following modifications, (N. H. Mann, pers. comm.). An overnight culture was diluted 1:50 in 50 ml prewarmed (37°C) LB in a 250 ml flask. The culture was then grown to an OD_{600} = 0.5 (*ca.* 3 x 10^8 organisms ml^{-1}). 40 ml of the culture was then placed in two prechilled universals and left to stand on ice for 10 min. Cells were then pelleted rapidly in an MSE multex bench top centrifuge (5,200 rpm, 30 sec). The supernatant was decanted and the pellet resuspended in an equal volume of ice cold 0.1 M $MgCl_2$. The cells were then pelleted as before, supernatant removed and the resulting pellet resuspended in 0.5 volume of ice cold 0.1 M $CaCl_2$ (Grade 1 $CaCl_2$ - Sigma No. c-3881). The cells were again pelleted as before, the supernatant removed, and the pellet resuspended in 0.05 volume of ice cold 0.1 M $CaCl_2$. After a minimum of one hour on ice, 200 µl aliquots of the competent cells were aliquoted into 1.5 ml Eppendorf tubes (Sarstadt Ltd.). DNA (50 ng saturates 200 µl of cells) was added to the cells, mixed gently and then left on ice for 30 min. These were then heat shocked at 42°C for 2 mins in a water bath and returned to ice for 15 min.

Two volumes of LB were then added to each tube, incubated for one hour at 37°C, dilutions made and cells plated out onto selective media.

During the construction of gene banks the Hanahan method was adopted (Hanahan, 1983).

2:8:2 *K. pneumoniae*.

The freeze-thaw method as described by Merrick *et al.* (1987) was adopted.

2:8:3 *M. capsulatus* (Bath).

A number of methods have been attempted. These are described in Chapter 7 (of this thesis).

2:9 Southern transfer of DNA.

The procedure described in Maniatis *et al.* (1982) was used routinely with the following modifications. Denaturation of the DNA in the agarose gel as well as neutralisation of the DNA in the gel matrix were both carried out for 30 min at room temperature. 20 x SSC was used instead of 10 x SSC as the transfer buffer.

2:10 Transfer of bacterial colonies to nitrocellulose and the binding of liberated DNA (Colony blots).

The procedure of Grunstein and Hogness (1975) as described in Maniatis *et al.* (1982) was used, with the following modifications. Large petri dishes, 20 cm x 20 cm, were used containing 500 ml LB agar with the appropriate antibiotic/s. Nitrocellulose (Hybond-C; Amersham) was placed onto the LB agar plates and colonies picked onto duplicate plates. After

the plates had been incubated at 37°C overnight, the master plate was stored at 4°C for up to 6 weeks and the nitrocellulose filter was treated as described in Maniatis *et al.* (1982).

2:11 Nick translation of DNA

The basic procedure of Maniatis *et al.* (1982) with the following modifications was used (C. J. Oakley, Pers. Comm.).

X µl	DNA in TE (*ca.* 250 ng)
1 µl	1 mM dATP
1 µl	1 mM dTTP
1 µl	1 mM dCTP
1 µl	^{32}P-dGTP (Amersham; specific activity 3000 Ci mmol^{-1} at a concentration of 10 µCi µl^{-1})
4 µl	10 x Nick translation buffer
0.8 µl	DNA polymerase I
1 µl	DNAse I (1 µl of 1 mg/ml stock in 50 µl dH_2O then 1 µl of above in 50 ml dH_2O)
Y µl	dH_2O

X and Y are adjusted to give 20 µl final volume.

The above constituents were placed in a screw top 1.5 ml Eppendorf tube, spun in a MSE microcentaur at high speed to mix the contents and then incubated at 15°C for 3 hours. The reaction was then terminated by the addition of 5 µl 0.25 M EDTA (pH 8.0) and the nick translated probe separated from unincorporated dNTPs using a G50 sephadex column. The

labelled DNA was then stored at -20°C until required when it was denatured by boiling for 15 min immediately prior to use.

2:12 Hybridization of Southern filters and colony blots.

The procedure described in Maniatis *et al.* (1982) was essentially followed with the following modifications. Nitrocellulose filters from either Southern or colony blotting were placed dry in heat-sealed Sterilin bags containing pre-hybridization solution (6 x SSC, 1 x Denhardts solution (Denhardt, 1966) and 10 mg ml^{-1} sheared, heat-denatured Herring sperm DNA). After pre-hybridization in a water bath at 65°C for 2 hours, the pre-hybridization solution was replaced with fresh pre-hybridization solution supplemented with the ^{32}P-labelled probe. Hybridization was carried out for a minimum of 18 hours at 65°C, followed by a variety of stringency washes employing previously published guidelines (Marmur and Doty, 1962; Dove and Davidson, 1962; Bonner *et al.*, 1973) to estimate the percentage DNA homology required for hybridization with *Methylococcus capsulatus* (Bath) DNA which has a G + C DNA ratio of 62.5% (Whittenbury and Krieg, 1984). For example, it was estimated that washing in 1.0 x SSC at 80°C requires greater than 70% homology between a *K. pneumoniae* probe and its target sequence in *M. capsulatus* DNA for continued hybridization.

2:13 Autoradiography.

Autoradiography was carried out at -70°C for ^{32}P-labelled material and at room temperature for ^{35}S-labelled material using Harmer film cassettes (with intensifying screens for ^{32}P) and Fuji RX X-Ray film. Autoradiograms were developed in Kodak LX-24 developer and fixed in Kodak FX-40 according to the manufacturers' instructions.

2:14 Construction, maintenance and screening of *N. capsulatus* (Bath) selective gene library.

DNA fragments of between 4.5 Kb and 5.5 Kb from a total *Eco*RI digest of *N. capsulatus* (Bath) genomic DNA (Section 2:5) were electroeluted (Section 2:7:8) from a preparative 0.7% (w/v) agarose gel. These DNA fragments were ligated into *Eco*RI cut, daphosphorylated pBR325 (see Figure 2:1) and transformed into *E. coli* HB101. 400 of the resulting ampicillin^R, tetracycline^R, chloramphenicol sensitive colonies were then colony blotted onto nitrocellulose and onto a master plate which was stored at 4°C. This colony blot was then probed with radiolabelled pAM51 0.86 Kb *Eco*RI insert at a stringency requiring 60% homology for a G + C ratio of 62.5%.

2:15 Construction and maintenance of a cosmid library of *N. capsulatus* (Bath).

The following cosmid gene library was constructed and kindly donated by C. J. Oakley (University of Warwick).

Genomic DNA from *N. capsulatus* (Bath) was partially digested with the restriction endonuclease *Sal*I to produce the greatest proportion of fragments in the size range, 18 Kb to 28 Kb. All DNA fragments in this size range were then electroeluted from a preparative agarose gel and ligated into the *Sal*I site of the cosmid pVK100 (see Figure 2:1). An Amersham Int. lambda *in vitro* packaging kit was used to introduce recombinant cosmids into *E. coli* HB101. 400 recombinant clones were colony blotted onto nitrocellulose.

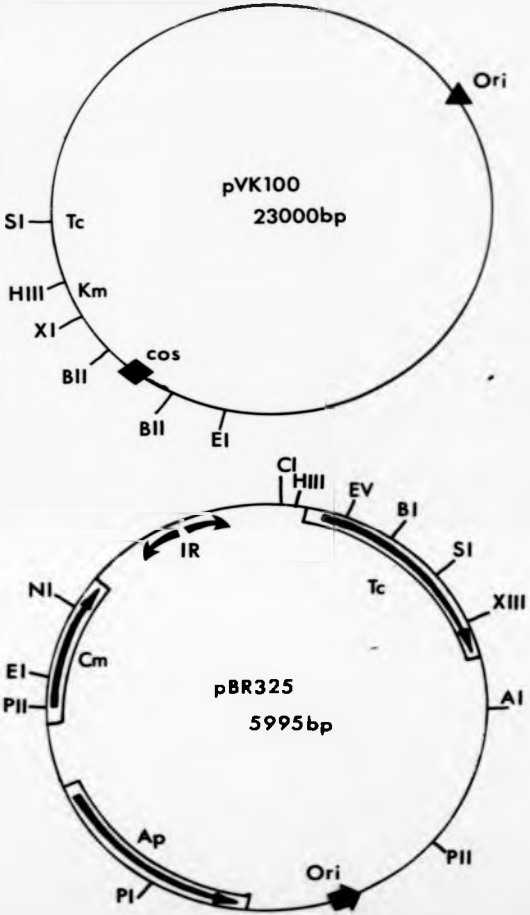

Ori
pVK100
23000bp
SI
Tc
HIII
Km
XI
cos
BII
BII
EI
CI
HIII
EV
BI
SI
XIII
AI
PII
Ori
PI
Ap
NI
EI
PII
Cm
IR
Tc
pBR325
5995bp

Figure 2:1 Cloning vectors pBR325 and pVK100.

Multicopy cloning vector pBR325 - Bolivar, (1978)

Commid cloning vector pVK100 - Knauf and Nester, (1982)

Key:

E1	*Eco*RI
N1	*Nco*I
P11	*Pvu*II
C1	*Cla*I
H111	*Hin*dIII
EV	*Eco*RV
B1	*Bam*HI
S1	*Sal*I
X111	*Xma*III
A1	*Ava*I
P1	*Pst*I
B11	*Bgl*II
X1	*Xho*I
IR	Inverted repeat
ORI	Origin of replication
COS	Cohesive site
Tc	tetracycline resistance gene
Ap	ampicillin resistance gene
Km	kanamycin resistance gene
Cm	chloramphenicol resistance gene

2:16 Expression analysis.

2:16:1 Maxicell system.

Plasmid encoded proteins were identified using a modified 'maxi' cell system of Sancar *et al.*, 1979 and Stoker *et al.*, 1984 devised by D. Gill (pers. comm.).

E. coli CSH26ΔF6 was transformed with the desired plasmid. CSH26ΔF6 derivatives were grown overnight in K-medium containing the appropriate antibiotics. 150 μl of this culture was then inoculated into a 250 ml flask containing 15 ml fresh K-medium plus antibiotics and incubated in a shaking 37°C water bath. When a culture density of A_{600} = 0.5 (2 x 10^8 cells ml^{-1}) was attained, cells were placed on ice. 10 ml of cells were UV-irradiated using gentle agitation in a Petri dish and exposed to a dose of *ca.* 50 J/M^2. These cells were then transferred to a sterile 250 ml flask, fresh cycloserine (200 μg ml^{-1}) added and the culture incubated for 14-16 hours, at 37°C with shaking. Cells were transferred to sterile universals, collected by centrifugation in an MSE Multex (4,000 rpm, 5 min, room temperature), washed twice with Hershey salts (5 ml) and finally resuspended in Hershey medium (5 ml) containing cycloserine (200 μg ml^{-1}). Following incubation at 37°C, with shaking for one hour, in a 250 ml flask, 40 μCi ^{35}S-methionine (specific activity = 1490 Curico $mmol^{-1}$) were added and incubation continued for a further hour. Cells were then harvested by centrifugation, washed twice with 10 mM Tris (pH 8.0) (5 ml), resuspended in 50 μl 10 mM Tris (pH 8.0) and an equal volume of 2 x PAGE (lysis) sample buffer added. Samples were boiled for 5 min prior to electrophoresis. Plasmid encoded proteins were identified by fluorography of polyacrylamide gels (Section 2:19).

2:16:2 DNA-directed *in vitro* transcription-translation (Zubay) coupled system.

A prokaryotic DNA-directed *in vitro* transcription-translation kit (N.380 Amersham) was used according to the manufacturers recommendations, using ^{35}S-methionine as the labelled amino acid. This system is based on the method of Zubay (see Pratt, 1984). Plasmid encoded proteins were identified by fluorography of electrophoresed samples on polyacrylamide gels (Section 2:19).

2:17 Mutagenesis.

2:17:1 Tn5 mutagenesis-transduction with λ_{delv}.

A modified method of de Bruijn and Lupski, (1984) (pers. comm. - J. C. D. Hinton) was used in an attempt to construct pBR325::*glnA*$_{Mc}$::Tn5. A fresh overnight culture of CSH26AF6/pDCI was diluted 1:20 into 10 ml of LB ApTc + 10 mM $MgSO_4$ + 0.2% (w/v) maltose. This was incubated at 37°C until a culture density of A_{440} = 0.8 was attained. Cells were centrifuged in a MSE Multex (5,000 rpm, 5 min) and resuspended in 1 ml of the same media. A portion was removed as a control for spontaneous Km resistance. 4 x 10^8 pfu of λ_{delv} (kindly donated, J. C. D. Hinton) was added, and the cells were incubated (static) at 30°C for 2 hours. Aliquots were then spread on LB agar containing ampicillin, tetracycline and kanamycin (300 μg ml^{-1}) and incubated at 37°C for 24-36 hours. Ap^{R}Tc^{R}Km^R (200-300 colonies/plate) were scraped off 10 plates and plasmid DNA isolated. This plasmid DNA was used to transform competent cells of *E. coli* ET8894 and Ap^{R}Tc^{R}Km^R transformants selected on LB agar ApTcKm plates supplemented with 0.2% glutamine. The Ap^{R}Tc^{R}Km^R clones were then used for further studies, as described in Results (Chapter 4).

2:17:2 Site directed mutagenesis - Ω fragment.

Construction of pDC2Ω (Table 2:2) was carried out using the Ω fragment of Prentki and Krisch, (1984). pDC2 was partially digested with the restriction endonuclease *Hin*dIII, dephosphorylated to prevent self ligation, mixed and ligated to the 2 Kb *Hin*dIII Ω fragment (prepared as in Section 2:7:8) of pHP45Ω. The resulting ligation mixture was then transformed into *E. coli* TH16 and plated onto LB agar ApTc + 0.2% (w/v) glutamine. Transformants were then replica plated onto M9 agar ApTc 0.1% (w/v) NH_4Cl and M9 agar ApTc 0.2% (w/v) glutamine. Mini plasmid preparations were then carried out on transformants which were found to require glutamine. The resulting plasmids were digested with restriction endonuclease *Sal*I and analysed by agarose gel electrophoresis in order to identify the required recombinant (pDC2::Ω).

2:17:3 Marker exchange mutagenesis.

The three way filter mating procedure described by Toukdarian and Lidstrom (1984) was used except NMS agar plates were supplemented with 0.1% (w/v) glutamine instead of 0.1% (w/v) NH_4Cl. Antibiotic selection for *M. capsulatus* (Bath) recipients was with streptomycin (20 $\mu g\ ml^{-1}$). Filters used in these bacterial matings were 0.22 μm Millipore filters.

2:18 DNA sequencing.

The dideoxynucleotide chain termination method of DNA sequencing (Sanger *et al.*, 1977; Sanger *et al.*, 1980) was employed.

2:10:1 Template preparation.

Didoxy sequencing reactions were carried out using defined restriction fragments cloned in M13tg130 and tg131 vectors. Template preparation was essentially as described by Bankier *et al.* (1986) with the following modifications. *E. coli* TG1 was used as the host for M13 recombinant propagation. After propagation of phage for 5 hours, cultures were transferred to 1.5 µl Eppendorf tubes and centrifuged in a MSE Microcentaur (20 min, high speed, room temperature). After careful transfer of supernatant to a fresh Eppendorf tube, PEG (6,000 m.wt., 20% w/v) (150 µl) was added to each supernatant, vortexed briefly, and left standing at room temperature for a minimum of 10 min. The supernatant/PEG solution was centrifuged to remove PEG. Any residual PEG was removed after a brief 30 sec respin. The resulting phage pellet was then resuspended in 100 µl TE, allowed to stand at room temperature for 10 min and 50 µl TE saturated phenol added, vortexed well and centrifuged for 5 min at room temperature. 90 µl of the resulting aqueous layer was removed and placed into a fresh Eppendorf tube containing 7.5 M NH_4 acetate (45 µl) and 100% ethanol (200 µl), vortexed, and placed at -20°C overnight. After precipitation, the vacuum-dried pellet was redissolved in 20 µl TE and stored at -20°C.

2:10:2 Didoxy sequencing

The procedure described in the Bethesda Research Laboratories (BRL) M13 Cloning/Didoxy Sequencing Manual was essentially followed with the following modifications. For the template-primer annealing reaction, 2.5 µl of template DNA was mixed with 1.5 µl sequencing buffer (5x), 0.5 pmol M13 17-base primer (kindly supplied by H. Raybutt) and dH_2O up to 10 µl final volume. Annealing was accomplished at 55°C for 1-2 hours. All

subsequent steps i.e. sequence reactions and denaturing were carried out in microtitre trays. Denaturation was carried out in an oven (Mini/696/Clad) at 80°C for 15 min and placed immediately on ice prior to loading the whole of each reaction mixture on a 6% (w/v) polyacrylamide, Tris-borate-urea sequencing gel.

2:18:3 Buffer gradient acrylamide gel electrophoresis.

The procedure described by Bankier *et al.* (1986) was followed using either 50 cm x 20 cm or 100 cm x 20 cm vertical sequencing gel apparatus (Raven). The power supply used for electrophoresis was an LKB Model 2103 power pack.

2:18:4 Sequence analysis.

All DNA sequence derived from this work was recorded and analysed using the Microgenie sequence analysis program of Queen and Korn, 1986 using an IBM PCAT computer.

2:19 Polyacrylamide gel electrophoresis (PAGE).

2:19:1 Non-denaturing tube gels.

A modified method for the analysis of proteins on non-denaturing disc gels (Gabriel, 1971) was used. A resolving gel concentration of 5% (w/v) was used with samples containing up to 50 μg of protein were loaded onto each gel and electrophoresis carried out at 4°C (2.5 mA per gel). After electrophoresis, the gel was either Coomassie stained for protein (see Section 2:19:3:1) or activity stained for glutamine synthetase activity (see Section 2:19:3:2).

2:19:2 Slab gels.

The method was based on that of O'Farrell (1975) and used a discontinuous buffer system as described by Laemmli (1970). 3 M Tris-HCl pH 8.8 buffer was used in preparation of the resolving gel and 0.5 M Tris-HCl pH 6.8 buffer was used in the preparation of the stacking gel. The running buffer used was Tris-glycine (0.025 M Tris base, 0.192 M glycine). Exponential gradient gels from 4% to 15% (w/v) and single percentage acrylamide gels of 10.5% (w/v) were used. Electrophoresis was carried out at a constant current of 15 mA at 4°C using a BRL vertical gel electrophoresis system (20 cm x 20 cm gel plates). For sodium dodecyl sulphate (SDS) electrophoresis, the stacking gel, resolving gel and running buffer contained SDS at 0.1% (w/v) and 2-mercapto-ethanol at 1 mM.

Cell-free extracts for non-denaturing gels were prepared in 10% (w/v) sucrose. Extracts for SDS-PAGE were boiled for 5 min in sample buffer (0.125 M Tris-HCl pH 6.8, 10% (w/v) sucrose and 40% (w/v) SDS). Prior to loading onto the gel, samples were mixed with bromophenol blue tracking buffer (10 µl 0.1% (w/v) bromophenol blue per 0.5 ml of sample).

Protein standards used were Phosphorylase b, M_r 94,000; Bovine serum albumin, M_r 67,000; Ovalbumin, M_r 43,000; Carbonic anhydrase, M_r 30,000; Soybean trypsin inhibitor, M_r 20,100; and α-Lactalbumin, M_r 14,000. Supplied by Pharmacia.

2:19:3 Gel staining.

2:19:3:1 Coomassie Blue stain.

Gels were stained for 5 hours in 0.1% Coomassie Blue R in 10% (v/v) acetic acid and 40% (v/v) methanol. Gels were destained in the same solvent for 4-8 hours.

2;12;3;2 Activity stain.

Glutamine synthetase activity was located in non-denaturing tube and slab gels by incubation at 45°C with the γ-glutamyltransferase assay mixture: (tube gels (50 ml), slab gels (250 ml) containing imidazole-HCl pH 7.15 (133 mM), hydroxylamine-HCl (18 mM) and L-glutamine (20 mM). After 20 minutes at the desired temperature, the gels were removed and placed in the γ-glutamyltransferase 'stop mix' solution until the characteristic brown band of γ-glutamylhydroxamate appeared in the gel (Kleinschmidt and Kleiner, 1978). The gels were then photographed.

2;12;4 Photography.

Coomassie Blue stained gels were routinely photographed using a Pentax SP500 camera with Kodak Panatomic X film (ASA 32).

Activity stained gels were also photographed using a Pentax SP500 camera with Kodak Colour film (ASA 100).

2;12;5 Fluorography.

Following electrophoresis, gels were fixed in glacial acetic acid (AR grade) (200 ml) for 5 min, impregnated in 2, 5-diphenyl-oxazole (PPO) (40 g) in glacial acetic acid (190 ml) for 90 min, washed in water for 30 min and then dried in a Bio-Rad model 1125 B gel drier at 60°C for 2 hours. The dried gel was then subjected to fluorographic exposure using Fuji RX X-ray film.

^{14}C-molecular weight markers used were supplied by Amersham International:-

[^{14}C] methylated Lysozyme	M_r 14,300
[^{14}C] methylated Carbonic anhydrase	M_r 30,000
[^{14}C] methylated Ovalbumin	M_r 46,000
[^{14}C] methylated Bovine serum albumin	M_r 69,000
[^{14}C] methylated Phosphorylase b	M_r 92,000
[^{14}C] methylated Myosin	M_r 200,000

2:20 Preparation of cell free extracts.

Cell free extracts of *Methylococcus capsulatus* (Bath) for glutamine synthetase assays were prepared as follows: whole cells of *M. capsulatus* (Bath) grown in NS, NMS or AMS media from continuous culture were donated by J. C. Murrell. These cells were harvested by centrifugation (6,000 rpm, 10 min, 4°C), washed with 25 mM imidazole-hydrochloride buffer (pH 7.15 at 45°C) and finally resuspended in 25 mM imidazole-hydrochloride buffer (pH 7.15 at 45°C). Cell-free extracts were prepared by sonication of whole cells using an MSE sonicator. Sonication was carried out on ice with 3 x 6 sec 18 micron peak to peak bursts with a 30 second cooling period between each burst. After sonication extracts were centrifuged (10 min, high speed, 4°C). Supernatants were carefully removed, drop-frozen in liquid nitrogen and stored at -70°C.

E. coli cell-free extracts were prepared as above except whole cells were derived from 1 litre batch cultures harvested at an A_{660} of 0.7 and resuspended in imidazole-hydrochloride buffer (pH 7.15 at 37°C).

Protein concentration was assayed using a modified method of Lowry *et al.* (1951) as described by Herbert *et al.* (1971).

2:21 Enzyme assays.

2:21:1 Spectrophotometric procedures.

All spectrophotometry was carried out using a Unicam SP1800 UV recording spectrophotometer.

2:21:2 γ glutamyltransferase assay.

A modified procedure of Bender *et al.* (1977) as described by Murrell and Dalton (1983(a)) was used. The assays were carried out either at 37°C or 45°C.

Cloning of the structural gene for glutamine synthetase (*glnA*) from *N. capsulatus* (Bath).

3:1. Introduction.

The structural gene for glutamine synthetase (*glnA*) has been cloned from a variety of bacterial genera (see Table 1:3; Chapter 1). Due to the availability of these cloned genes for use as gene probes, a heterologous hybridization approach was employed in order to identify and isolate the relevant DNA fragment encoding glutamine synthetase from *N. capsulatus* (Bath).

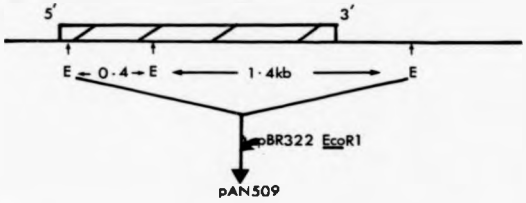
3:2. Results.

3:2:1. Heterologous hybridization studies using a cloned cyanobacter *glnA* gene as DNA probe.

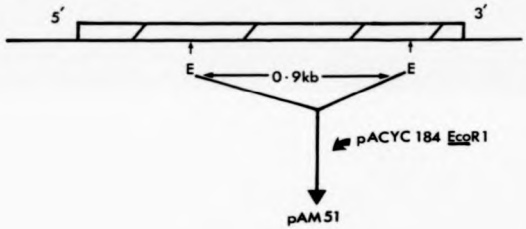
N. capsulatus (Bath) genomic DNA was digested to completion with a variety of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at low stringency (allowing for approximately 60% bp mismatch) with the [α-^{32}P] dGTP labelled *Anabaena* 7120 *glnA* gene (see Figure 3:1). The resulting autoradiograph is shown in Figure 3:2.

No detectable homology was observed between the *Anabaena* 7120 *glnA* gene probe with any *N. capsulatus* sequences. Subsequent hybridization studies utilizing the *Anabaena 7120 glnA* gene revealed no apparent homology to any *N. capsulatus* (Bath) sequences, even under conditions of very low stringency (<20% homology, >80% bp mismatch).

pAN509 EcoR1 inserts (Anabaena 7120 glnA)



pAM51 EcoR1 insert (K. pneumoniae glnA)



0·5kb

Figure 3:1 DNA probes used in this study

Relevant fragments were excised from plasmid vector, fractionated by agarose gel electrophoresis, eluted from gel matrix and nick translated ready for use as hybridization probe.

Key:

E = *Eco*RI

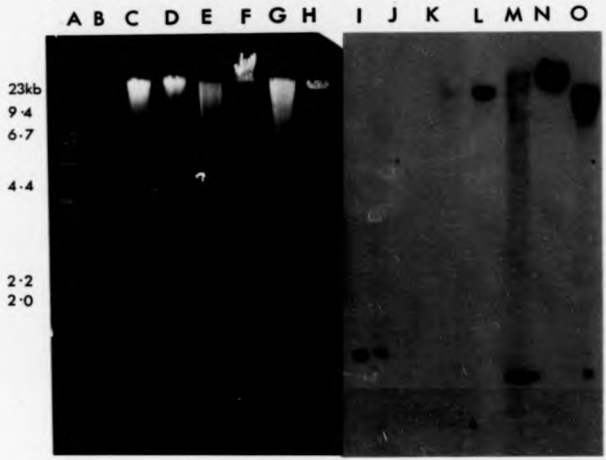
Figure 3:2 Examination of *M. capsulatus* (Bath) genomic DNA for homology with the *Anabaena* 7120 *glnA* gene.

M. capsulatus (Bath), *Anabaena* 7120 and *E. coli* HB101 genomic DNAs were restricted as indicated, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose. The resulting blot was probed with the 0.4 kb and 1.4 kb *Eco*RI inserts of pAN509 (see Figure 3:1) at low stringency and subjected to autoradiography.

Key to tracks:

A	*M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
B	*M. capsulatus* (Bath) genomic DNA restricted with *Sal*I
C	*M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII
D	*Anabaena* 7120 genomic DNA
E	*Anabaena* 7120 genomic DNA restricted with *Eco*RI
F	*E. coli* HB101 genomic DNA
G	*E. coli* HB101 genomic DNA restricted with *Sal*I
H	λ DNA restricted with *Hin*dIII

Tracks I to O are autoradiographs of corresponding tracks A to G probed with the *Anabaena* 7120 *glnA* gene probe (pAN509 0.4 kb and 1.4 kb *Eco*RI inserts).


A B C D E F G H I J K L M N O
23kb
9·4
6·7
4·4
2·2
2·0

3:2:2. Heterologous hybridization studies using a cloned *Klebsiella pneumoniae glnA* gene probe.

A similar approach to that described above was employed, utilizing a cloned internal *Eco*RI fragment of the *glnA* gene from *K. pneumoniae* M5al as a heterologous DNA probe (see Figure 3:1).

N. capsulatus (Bath) genomic DNA was digested to completion with a variety of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed initially at low stringency (allowing 60% bp mismatch) with ^{32}P-labelled *K. pneumoniae glnA* fragment. The resulting autoradiograph patterns are shown in Figure 3:3 and summarized in Table 3:1.

Progressively more stringent washes indicated an approximate 65% homology (approximate 35% bp mismatch) between the *K. pneumoniae* internal *glnA* gene fragment and its target *N. capsulatus* (Bath) sequences.

3:2:3. Cloning and characterization of the *glnA* gene from *N. capsulatus* (Bath).

The 5.2 Kb *Eco*RI fragment of *N. capsulatus* (Bath) genomic DNA previously shown to contain homologous sequences to the *K. pneumoniae glnA* gene probe (see Figure 3:3), was chosen for isolation. This fragment was selected so as to optimize the chances of isolating the entire *N. capsulatus* (Bath) *glnA* gene including its control region. The cloning strategy adopted is detailed in Figure 3:4 which involved the construction of a selective gene bank in the *Eco*RI site of pHR325, the resulting library being screened by hybridization.


A B C D E F G H I J K
23kb
9·4
6·7
4·4
2·2
2·0
0·6

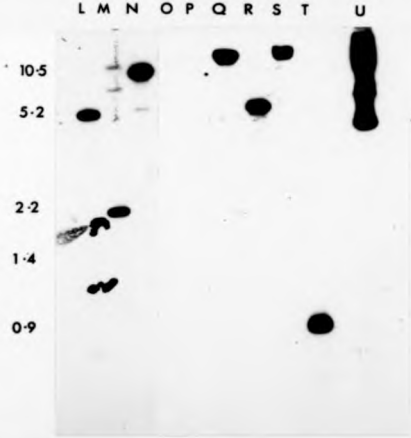

L M N O P Q R S T U
10·5
5·2
2·2
1·4
0·9

Figure 3:3 Examination of *N. capsulatus* genomic DNA for homology with the *Klebsiella pneumoniae* M5al *glnA* gene.

N. capsulatus (Bath), *Anabaena* 7120 and *E. coli* HB101 genomic DNAs were restricted as indicated, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose. The resulting blot was probed at low stringency with the 0.9 kb *Eco*RI insert of pAM51 (see Figure 3:1) and subjected to autoradiography.

Key to tracks:-

A	*N. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
B	*N. capsulatus* (Bath) genomic DNA restricted with *Sal*I
C	*N. capsulatus* (Bath) genomic DNA restricted with *Hind*III
D	*Anabaena* 7120 genomic DNA
E	*Anabaena* 7120 genomic DNA restricted with *Eco*RI
F	*E. coli* HB101 genomic DNA
G	*E. coli* HB101 genomic DNA restricted with *Sal*I
H	*K. pneumoniae* genomic DNA
I	*K. pneumoniae* genomic DNA restricted with *Eco*RI
J	pAM51 DNA
K	λ DNA restricted with *Hind*III

Tracks L to U are autoradiographs of corresponding tracks A to K probed with the 0.9 kb pAM51 *Eco*RI fragment.

TABLE 3:1.

Sizes of *M. capsulatus* (Bath) genomic DNA restriction fragments that show homology to the *K. pneumoniae* internal *glnA* gene fragment.

Restriction endonuclease	*EcoRI*	*SalI*	*HindIII*
Fragment sizes (kb)	5.2	2.2	10.0

This table summarizes the data shown in Figure 3:3.

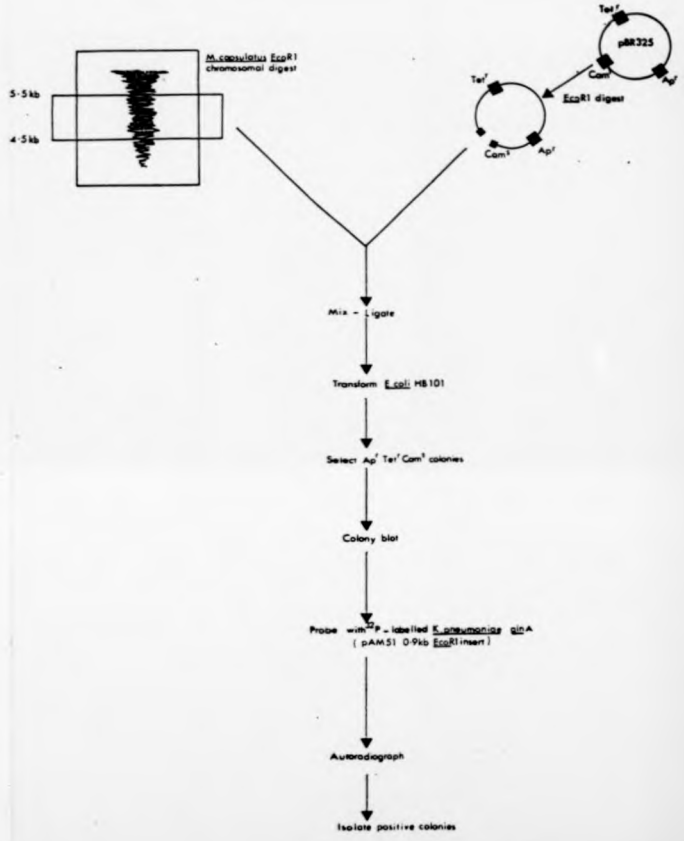

M.capsulatus EcoR1 chromosomal digest
5·5 kb
4·5 kb
pBR325
Tet^r
Cam^r
Ap^r
EcoR1 digest
Mix - Ligate
Transform E.coli HB101
Select Ap^r Tet^r Cam^s colonies
Colony blot
Probe with ^32P - labelled K.pneumoniae glnA
(pAM51 0·9kb EcoRI insert)
Autoradiograph
Isolate positive colonies

Figure 3:4 Cloning strategy adopted for the isolation of the *M. capsulatus* (Bath) *glnA* structural gene.

The 5.2 kb *Eco*RI fragment from *M. capsulatus* (Bath) genomic DNA was chosen for isolation as shown.

Due to the strong sequence homology between the *K. pneumoniae glnA* gene probe and the resident *glnA* gene of the surrogate *Escherichia coli* HB101 host (see Figure 3:3) used to construct the gene library, the amplifiable high copy number vector pBR325 (Figure 2:1; Chapter 2) was used to optimize the autoradiograph signal of any positive clone over the background signal resulting from this homology.

Screening of the library was carried out with ^{32}P-labelled *K. pneumoniae glnA* gene probe. The resulting autoradiograph, Figure 3:5, shows two clones clearly above background, designated clone 1 and clone 2. In addition, the *K. pneumoniae glnA* gene probe also exhibited homology to the *Anabaena* 7120 cloned *glnA* gene present as a control.

Subsequent hybridization analysis of plasmid DNA isolated from clones 1 and 2, probed with the *K. pneumoniae glnA* gene probe revealed that, unusually, both recombinants harboured dual *Eco*RI DNA fragment inserts, but had the required 5.2 *Eco*RI fragment in common (see Figure 3:6). Subcloning of this 5.2 Kb *Eco*RI fragment into pBR325 was carried out by subsequent isolation of clone 2 plasmid DNA, restricting to completion with *Eco*RI, inactivation of *Eco*RI by heat (70°C, 15 min), religation, transformation into *E. coli* HB101 and $Ap^{R}Tc^{R}Cm^{S}$ clones isolated. Plasmid DNA's from five $Ap^{R}Tc^{R}Cm^{S}$ clones (designated 2/1 to 2/5) were isolated, restricted with *Eco*RI, fractionated by agarose gel electrophoresis, Southern blotted and probed with the *K. pneumoniae glnA* gene probe. Clone 2/5 was shown to harbour a single 5.2 Kb *Eco*RI DNA fragment which exhibited strong homology to the *K. pneumoniae glnA* gene probe (see Figure 3:7). This clone was chosen for further analysis and was redesignated pDC1.

pDC1 was mapped with respect to several restriction endonucleases (Figures 3:8, 3:9 and summarized in Figure 3:10). In order to verify that the cloned 5.2 Kb *Eco*RI fragment was derived from *M. capsulatus* (Bath) genomic DNA and that no rearrangements had occurred during the


clone 1
clone 2
A
B
C

Figure 3:5 Screening a selective *M. capsulatus* (Bath) gene bank for *glnA* sequences.

The cloning of the *M. capsulatus* (Bath) *glnA* structural gene was carried out as outlined in Figure 3:4. The resulting colony blot was probed at a stringency allowing 40% bp mismatch with the *K. pneumoniae* M5al *glnA* gene fragment (pAM51 0.9 kb *Eco*RI fragment). The figure shows two clones (clones 1 and 2) clearly above background.

Key to controls:

A HB101 harbouring pAM509

B HB101 harbouring pBR325

C HB101 harbouring pAM51

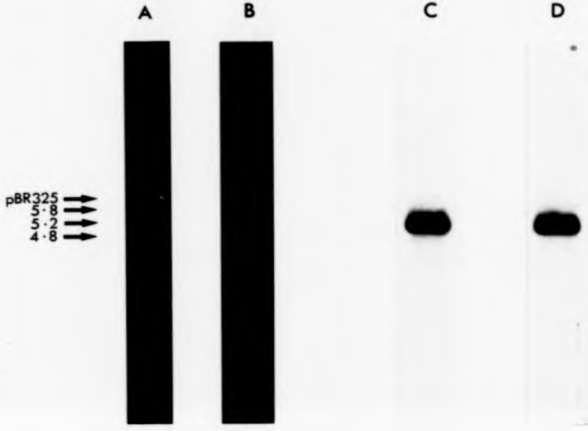

A
B
C
D
pBR325
5·8
5·2
4·8

Figure 3:6 Verification that clone 1 and clone 2 contain inserts exhibiting homology to the *K. pneumoniae glnA* gene probe.

Plasmid DNA from clones 1 and 2 were isolated, restricted with *Eco*RI to separate vector from inserts and fractionated by agarose gel electrophoresis. The resulting DNA fragments were transferred by Southern blotting onto a nitrocellulose filter, probed with the *K. pneumoniae glnA* fragment (pAM51 0.9 kb *Eco*RI fragment) at a stringency allowing 40% bp mismatch, and subjected to autoradiography.

Key to tracks:

A Clone 1 restricted with *Eco*RI

B Clone 2 restricted with *Eco*RI

C Autoradiograph of Clone 1 probed with pAM51 0.9 kb *Eco*RI fragment

D Autoradiograph of Clone 2 probed with pAM51 0.9 kb *Eco*RI fragment

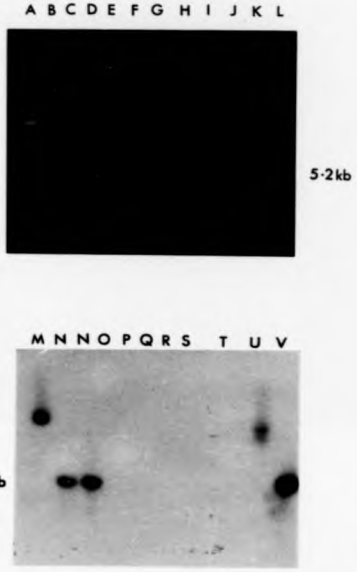
Figure 3:7 Construction and Isolation of pDC1.

Clone 2 plasmid DNA was restricted with *Eco*RI, religated, transformed into *E. coli* HB101 and plasmid DNAs from five Ap^R Tc^R Cm^S clones isolated, restricted with *Eco*RI, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose. This blot was probed with *K. pneumoniae glnA* gene fragment (pAM51 0.9 kb *Eco*RI fragment) and subjected to autoradiography.

Key to tracks:

A	λ DNA restricted with *Hin*dIII
B	Clone 2/1
C	Clone 2/1 restricted with *Eco*RI
D	Clone 2/1 restricted with *Eco*RI
E	Clone 2/2
F	Clone 2/2 restricted with *Eco*RI
G	Clone 2/3
H	Clone 2/3 restricted with *Eco*RI
I	Clone 2/4
J	Clone 2/4 restricted with *Eco*RI
K	Clone 2/5
L	Clone 2/5 restricted with *Eco*RI

Tracks M to V are autoradiographs of corresponding tracks B to L probed with *K. pneumoniae glnA* gene fragment (pAM51 0.9 kb *Eco*RI fragment).


A B C D E F G H I J K L
5·2kb
M N N O P Q R S T U V
5·2kb

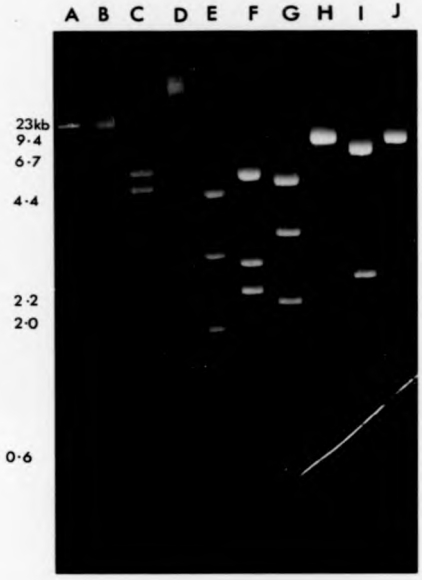

A B C D E F G H I J
23kb
9·4
6·7
4·4
2·2
2·0
0·6

Figure 3:8 Restriction endonuclease mapping of the *M. capsulatus* (Bath) *glnA* clone pDC1.

pDC1 plasmid DNA was digested to completion with a number of restriction endonucleases and the resulting DNA fragments were fractionated by agarose gel electrophoresis.

Key to tracks:

A	λ DNA restricted with *Hin*dIII
B	pDC1 DNA
C	pDC1 DNA restricted with *Eco*RI
D	pDC1 DNA restricted with *Xho*I
E	pDC1 DNA restricted with *Ava*I
F	pDC1 DNA restricted with *Bam*HI
G	pDC1 DNA restricted with *Sal*I
H	pDC1 DNA restricted with *Pst*I
I	pDC1 DNA restricted with *Hin*dIII
J	pDC1 DNA restricted with *Epn*I

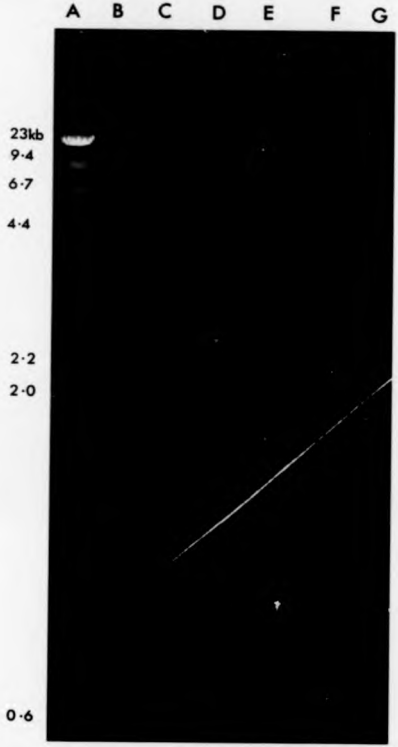

A B C D E F G
23kb
9·4
6·7
4·4
2·2
2·0
0·6

Figure 3:9 Restriction endonuclease mapping of pDC1.

pDC1 plasmid DNA was digested to completion with a number of restriction endonucleases and fractionated by agarose gel electrophoresis.

Key to tracks:

A	λ DNA restricted with *Hin*dIII
B	pDC1 DNA
C	pDC1 DNA restricted with *Eco*RI and *Hin*dIII
D	pDC1 DNA restricted with *Eco*RI and *Bam*HI
E	pDC1 DNA restricted with *Eco*RI and *Sal*I
F	pDC1 DNA restricted with *Eco*RI and *Kpn*I
G	pDC1 DNA restricted with *Eco*RI, *Ava*I and *Pst*I

pDC 1

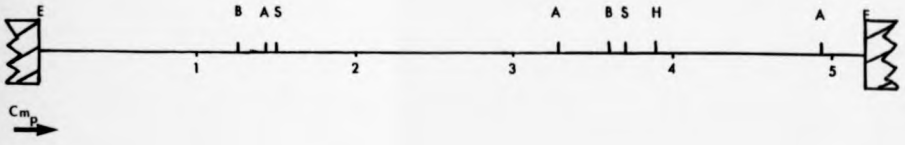


Figure 3:10 Endonuclease restriction map of pDC1 insert.

The 5.2 kb *M. capsulatus* (Bath) *Eco*RI fragment within pDC1 was mapped with respect to a number of restriction endonucleases.

Key:

E - *Eco*RI

A - *Ava*I

H - *Hin*dIII

S - *Sal*I

B - *Bam*HI

No : *Pst*I or *Xho*I sites present.

cloning procedure, the following checks were made. *N. capsulatus* (Bath) genomic DNA was digested to completion with a number of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted and then probed at high stringency with ^{32}P-labelled pDC1 5.2 Kb *Eco*RI insert. The resulting autoradiograph banding pattern (Figure 3:11) was compared to the restriction endonuclease map (Figure 3:10). The banding pattern of the restricted genomic DNA probed with pDC1 insert correlated with the cloned DNA, indicating that the pDC1 insert was derived from *N. capsulatus* (Bath) DNA and had not undergone any physical rearrangement.

The relative position of the *glnA* coding region within pDC1 was determined by probing various restriction digests of the pDC1 insert with the *K. pneumoniae glnA* gene probe (see Figure 3:12). The limits of the *N. capsulatus* (Bath) *glnA* gene within pDC1 are only approximate as the gene probe used was an internal fragment of the *K. pneumoniae glnA* gene (see Figure 3:12). The derivation of the limits and direction of transcription of the cloned *N. capsulatus* (Bath) *glnA* gene are described in detail in Chapter 4.

3:3. Discussion.

The structural gene for glutamine synthetase (*glnA*) has been cloned from *N. capsulatus* (Bath) by heterologous hybridization, utilizing an internal *glnA* gene fragment from *K. pneumoniae* M5al as gene probe. This method of cloning is similar to that undertaken by Fisher and his colleagues in the isolation of the *Anabaena* 7120 *glnA* gene and Carlson and his colleagues in the isolation of the GS1 structural gene (*glnA*) from *Bradyrhizobium japonicum*. The *Anabaena* 7120 *glnA* structural gene was isolated from an *Anabaena* 7120 *Hin*dIII λ gene bank, using a cloned *E. coli glnA* gene as heterologous hybridization probe after initial

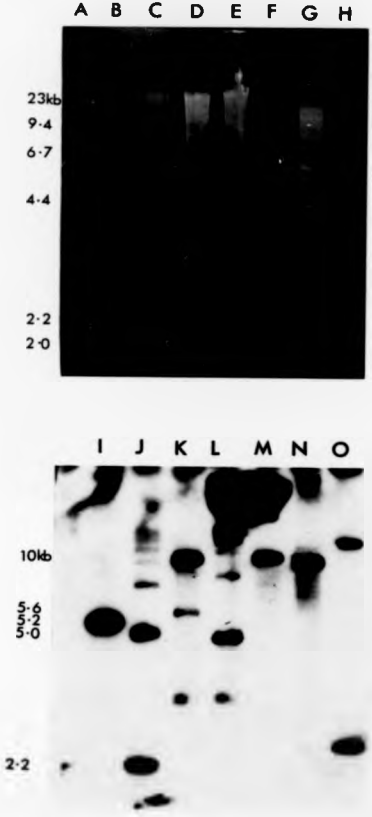

A B C D E F G H
23kb
9·4
6·7
4·4
2·2
2·0
I J K L M N O
10kb
5·6
5·2
5·0
2·2

Figure 3.11 Verification of the origin and continuity of the *M. capsulatus* (Bath) *glnA* clone pDC1.

M. capsulatus (Bath) genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter. This filter was then probed with the pDC1 5.2 kb *Eco*RI insert at high stringency (assuming an appropriate 5% bp mismatch).

Key to tracks:

A λ DNA restricted with *Hin*dIII

B *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI

C *M. capsulatus* (Bath) genomic DNA restricted with *Sal*I

D *M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII

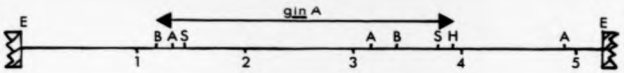
E *M. capsulatus* (Bath) genomic DNA restricted with *Kpn*I

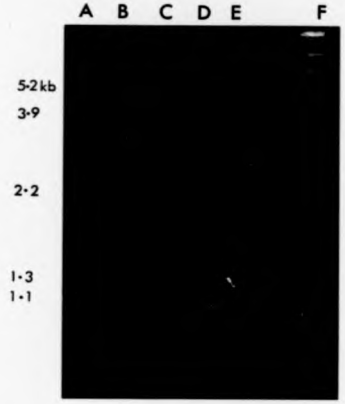
F *M. capsulatus* (Bath) genomic DNA restricted with *Xho*I

G *M. capsulatus* (Bath) genomic DNA restricted with *Pst*I

H *M. capsulatus* (Bath) genomic DNA restricted with *Cla*I

Tracks I to O are autoradiographs of corresponding tracks B to H probed with pDC1 5.2 kb *Eco*RI insert.


gln A
E
B A S
A B
S H
A
E
1
2
3
4
5


A B C D E F
5·2 kb
3·9
2·2
1·3
1·1

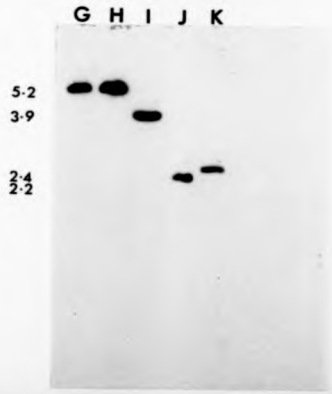

G H I J K
5·2
3·9
2·4
2·2

Figure 3:12 Determination of location of *glnA* within the 5.2 kb *EcoRI* insert of pDC1.

The 5.2 kb *EcoRI* insert of pDC1 was digested with a number of restriction endonucleases, fractionated by agarose gel electrophoresis and the resulting DNA fragments Southern blotted onto nitrocellulose. The resulting blot was probed at a stringency assuming an appropriate 40% bp mismatch with the *K. pneumoniae glnA* gene fragment (pAM51 0.9 kb *EcoRI* fragment).

Key to tracks:

A	pDC1 insert DNA
B	pDC1 insert DNA restricted with *PstI*
C	pDC1 insert DNA restricted with *HindIII*
D	pDC1 insert DNA restricted with *SalI*
E	pDC1 insert DNA restricted with *BamHI*
F	λ DNA restricted with *HindIII*

Tracks G to K are autoradiographs of corresponding tracks A to E probed with *K. pneumoniae glnA* gene fragment.

heterologous hybridization studies had identified a *glnA* homologue in the *Anabaena* 7120 chromosome (Fisher *et al.*, 1981). The *B. japonicum glnA* gene was also isolated from a phage λ library utilizing a fragment of the *E. coli glnA* gene as a heterologous hybridization probe (Carlson *et al.*, 1985).

The cloning strategy for the isolation of *glnA* from a number of other organisms has relied solely upon a 'shotgun' approach in which gene libraries are either conjugated or transformed into *E. coli* or *K. pneumoniae glnA* mutants. The desired recombinant is thus isolated by complementation of the *glnA* lesions in these organisms by returning them to glutamine independance. This method of cloning has been used to isolate the GS structural gene from the following organisms; *Rhizobium meliloti* GSI structural gene (Somerville and Kahn, 1983); *Bacillus subtilis* (Fisher *et al.*, 1984); *Thiobacillus ferrooxidans* (Barros *et al.*, 1985); *Rhizobium leguminosarum* GSI and GSII structural genes (Filser *et al.*, 1986); *Azotobacter vinelandii* (Toukdarian and Kennedy, 1986); *Bordetella pertussis* (Brownlie *et al.*, 1986); *Vibrio alginolyticus* (Maharaj *et al.*, 1986); *Clostridium acetobutylicum* (Usdin *et al.*, 1986) and *Agrobacterium tumefaciens* GSI and GSII structural genes, as well as a third *gln* locus, *glnT* (Rossbach *et al.*, 1988).

A heterologous hybridization approach was used to isolate the *glnA* gene from *M. capsulatus* (Bath) in these studies as it did not rely upon expression of the cloned gene for its initial isolation, unlike the 'shotgun' method. As no other obligate methanotroph gene had been expressed in a heterologous host prior to these studies, this method was employed to isolate the *glnA* gene from *M. capsulatus* (Bath).

Interestingly, the hybridization results obtained in this study indicated a lack of detectable homology between the *Anabaena* 7120 *glnA* gene probe used with any *M. capsulatus* (Bath) sequences. However, when the *K. pneumoniae glnA* internal fragment was used as the heterologous

hybridization probe, it showed a low but detectable homology to the *Anabaena* 7120 *glnA* sequences and relatively strong homology to specific *M. capsulatus* (Bath) sequences. Subsequent DNA sequence analysis (see Chapter 5) revealed a 59% nucleotide sequence homology between the *Anabaena* 7120 *glnA* gene and the *M. capsulatus* (Bath) *glnA* gene. Therefore, caution must be observed in the interpretation of hybridization data and the designation of hybridization stringencies as X% base pair mismatch etc.

Chapter 4

Expression analysis of the cloned *M. capsulatus* (Bath) *glnA* gene.

4:1 Introduction.

The future potential for the production of obligate methanotrophs with desirable characteristics for industry by genetic manipulation will depend, in part, on an understanding of gene expression in these important organisms. These studies were therefore designed to determine the expression and functionality of the *M. capsulatus* (Bath) *glnA* structural gene in a heterologous host, identify the protein product, and determine the nature of its regulation (i.e. Ntr dependance?) in a variety of strain backgrounds.

4:2 Results

4:2:1 Complementation analysis.

E. coli glutamine auxotrophs TH16 (*glnA*::Tn5) and ET8894 (*glnA-ntrC*)$^{\nabla}$ were each transformed with the recombinant pDC1 and pBR325 (control). Glutamine independent transformants were selected on M9 glucose minimal medium containing 0.1% (w/v) NH_4Cl as sole nitrogen source, ampicillin (100 $\mu g\ ml^{-1}$), tetracycline (20 $\mu g\ ml^{-1}$), (plus kanamycin (50 $\mu g\ ml^{-1}$) for Tn5 selection in TH16). Approximately equal numbers of *glnA*$^+$ and $Ap^R Tc^R$ transformants were obtained on minimal medium and LB agar containing ampicillin and tetracycline, when the recombinant pDC1 was used to transform competent cells of the *E. coli glnA* mutants TH16 and ET8894. However, no transformants were isolated on minimal medium containing ampicillin and tetracycline when pBR325 was used to transform the *E. coli*

glnA mutants TH16 and ET8894. This indicated that complementation of the *glnA* lesions present in these mutants was due to an intact and functional *M. capsulatus* (Bath) *glnA* structural gene, located within the 5.2 Kb *Eco*RI *M. capsulatus* (Bath) DNA fragment in pDC1.

The 5.2 Kb *Eco*RI insert of pDC1 was subsequently recloned in the opposite orientation in pBR325 by restriction of pDC1 with *Eco*RI followed by religation and transformation into *E. coli* HB101. The resultant plasmid was designated pDC2 and the orientation of the insert confirmed by restriction analysis. Subsequent transformation and complementation of the *glnA* lesions in TH16 and ET8894 (as described above) with pDC2, revealed that the cloned *M. capsulatus* (Bath) *glnA* gene was expressed from a regulatory region contained within the cloned DNA fragment and was not due to read through from a foreign (vector) promoter (e.g. the chloramphenicol acetyl transferase gene promoter of pBR325).

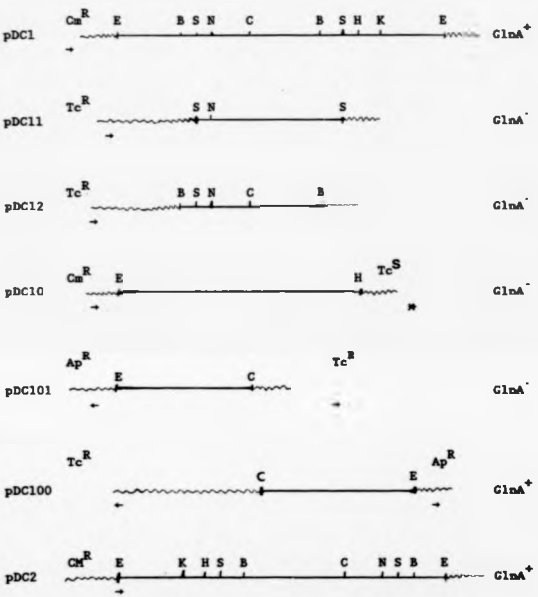
The recombinant plasmids pDC1 and pDC2, when transformed into the *K. pneumoniae glnA-ntrC* deletion mutant UNF1848, also corrected the glutamine auxotrophy of this organism to allow growth on NFDM agar containing 0.1% (w/v) NH_4Cl as sole nitrogen source.

4:2:2 **The localization of the *glnA* gene within pDC1.**

The localization of the *M. capsulatus* (Bath) *glnA* gene within pDC1 was determined by the isolation and characterization by complementation, of pDC1 and its derivatives (see Figure 4:1). The presence of additional restriction endonuclease cleavage sites within pDC1 (Figure 4:1) were also determined (data not presented).

Different DNA fragments were either deleted or subcloned into pBR325 or pBR327 and the GlnA phenotype conferred by these plasmids on TH16, determined. Figure 4:1 shows the restriction maps of the plasmids obtained and the GlnA phenotype they confer upon TH16.

Figure 4:1 Restriction map of pDC1 and derivative plasmids.



N.B. vectors used in the construction of these plasmids are stated in the text.

The plasmid pDC10 was generated by restriction of pDC1 with *Hin*dIII and subsequent religation, resulting in the isolation of a pDC1 tetracycline sensitive derivative due to the deletion of the 2.54 Kb *Hin*dIII fragment.

The plasmid pDC11 was generated by subcloning the 2.2 Kb internal *Sal*I fragment of pDC1 into the *Sal*I site of pAR325, in the same relative orientation as in the parental plasmid.

The plasmid pDC12 was generated by subcloning the internal 2.35 Kb *Bam*HI fragment of pDC1 into the *Bam*HI site of pAR325, in the same relative orientation as in the parental plasmid.

A double digest of pDC1 with *Eco*RI and *Cla*I, followed by subcloning of the resulting fragments into *Eco*RI-*Cla*I restricted pAR327 resulted in the isolation of pDC100 and pDC101 which contain the 2.7Kb and 2.5 Kb *Eco*RI-*Cla*I fragments respectively from pDC1. These two plasmids between them contain the entire 5.2 Kb *Eco*RI insert of pDC1.

The plasmid pDC2, as previously described, contains the 5.2 Kb *Eco*RI insert of pDC1 in the opposite orientation to that in pDC1.

Only the plasmid pDC1 and its derivatives, pDC2 and pDC100 complemented the *glnA* lesion in TH16 by correcting the glutamine auxotrophy of this organism. From these data therefore, it can be deduced that the entire *N. capsulatus* (Bath) *glnA* structural gene and its control region are located in pDC100, within the 2.7 Kb *Eco*RI-*Cla*I fragment derived from pDC1.

4:2:3 Identification of the cloned *N. capsulatus* (Bath) *glnA* gene product.

In order to identify the polypeptides encoded by pDC1 and to allocate one of these to the *glnA* gene, polypeptides synthesized by pDC1 and its derivatives were studied in a cell-free *in vitro* coupled transcription-translation system and also *in vivo*, using *E. coli* 'maxi' cells.

4:2:3:1 *In vitro* expression analysis.

An *E. coli* cell-free *in vitro* coupled transcription-translation system (see Chapter 2, Subsection 2:16:2) was used in these studies.

Following the *in vitro* transcription-translation reactions using ^{35}S-methionine as the labelled amino acid, the resulting proteins were electrophoresed on a 10% SDS denaturing polyacrylamide gel and fluorographed. The resulting fluorograph (Figure 4:2) shows that when DNA from pDC1 was used as a template, it directed the synthesis of only a single major polypeptide of M_r 60,000 in addition to vector (pBR325) specific polypeptides. When DNA from pDC2 was used as a template, it directed the synthesis of two major polypeptides of M_r 60,000 and M_r 42,000. Plasmid pDC100 directed the synthesis of a single major polypeptide of M_r 60,000, in addition to vector (pBR327) specific polypeptides.

When pDC10, pDC11 and pDC12 were assessed in this system only vector encoded polypeptides were observed. The pattern of polypeptides synthesized by all the recombinants in this system are summarized on Table 4:1.

4:2:3:2 *In vivo* expression analysis.

Plasmid-encoded proteins may be identified using the *E. coli* 'maxi' cell system (Sancar *et al.*, 1979). The plasmids pDC1, pDC2, pDC100 and pBR325 were transformed into the *E. coli* 'maxi' cell strain CSH26ΔF6 (Stoker *et al.*, 1984). The resulting transformants were allowed to synthesize proteins in the presence of ^{35}S-methionine as described in Chapter 2, Subsection 2:16:1. The *de novo* synthesized plasmid-encoded proteins were electrophoresed on a 10% SDS-denaturing polyacrylamide gel and visualized by fluorography. The resulting fluorograph (Figure 4:3)

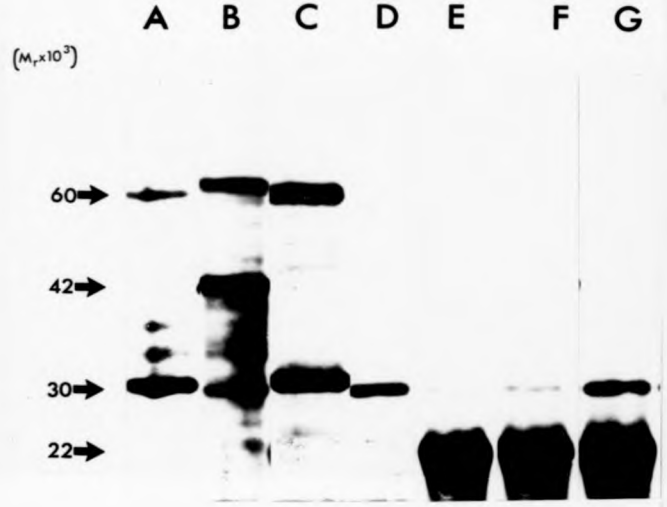

A
B
C
D
E
F
G
(Mr x10^3)
60
42
30
22

Figure 4.2 *In vitro* translation products of pDC1 and its derivatives.

The fluorograph shows the various ^{35}S-methionine labelled plasmid encoded products.
The M_r 30,000 and M_r 22,000 polypeptides correspond to β-lactamase and chloramphenicol acetyl transferase respectively of the vectors pBR325 and pBR327 (β-lactamase only).

Key to tracks:

A	pDC1	encoded products
B	pDC2	encoded products
C	pDC100	encoded products
D	pDC10	encoded products
E	pDC11	encoded products
F	pDC12	encoded products
G	pBR325	encoded products

Table 4:1 Pattern of *in vitro* polypeptide synthesis by pDC1 and derivatives.

Plasmid	M_r of Polypeptides			
	60,000 *glnA*	42,000	30,000 *bla*	26,000 *cat*
pDC1	++	-	++	-
pDC2	++++	++++	++	-
pDC10	-	-	++	-
pDC11	-	-	+	++++
pDC12	-	-	+	++++
pDC100	+++	-	++	-
pBR325	-	-	++	++++

The relative proportions of different polypeptides synthesized is indicated on a scale ++++ to + as judged by visual inspection.

- = not detected.

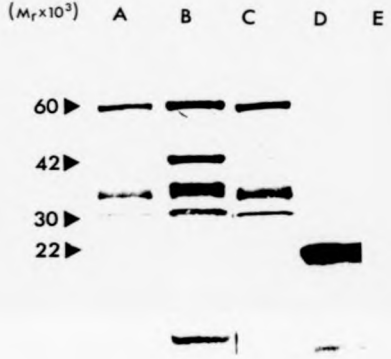

($M_r \times 10^3$)
A
B
C
D
E
60
42
30
22

Figure 4:3 **Identification of pDC1 and derivative plasmid-encoded proteins using the *E. coli* 'maxi' cell system.**

The fluorograph shows ^{35}S-methionine labelled polypeptide products of pDC1 and derivative plasmids using the *E. coli* 'maxi' cell system.

Key to tracks:

A	pDC1	encoded polypeptides
B	pDC2	encoded polypeptides
C	pDC100	encoded polypeptides
D	pBR325	encoded polypeptides
E	CSH26AF6	control

clearly shows the pattern of proteins synthesized by the *E. coli* 'maxi' cells harbouring pBR325 and additional proteins encoded by pDC1, pDC2 and pDC100. The pattern of polypeptides synthesized by all the recombinants in this system are summarized on Table 4:2.

The plasmids pDC1, pDC2 and pDC100 all directed the synthesis *in vivo* (as well as *in vitro*), of a major polypeptide with an apparent M_r of 60,000 as well as vector encoded polypeptides. In addition, pDC2 also directed the synthesis *in vivo* (as *in vitro*) of a major polypeptide of M_r 42,000. This polypeptide is unique to pDC2 and may be the result of a fusion between part of a 'vector' gene and a gene carried in the cloned fragment (see Section 4:2:4). The M_r 60,000 polypeptide encoded by all the GlnA$^+$ plasmids pDC1, pDC2 and pDC100 has an identical M_r to the reported *N. capsulatus* (Bath) GS monomer as identified by SDS-PAGE of the purified *N. capsulatus* (Bath) GS (Murrell and Dalton, 1983(c)). The M_r 60,000 polypeptide encoded by these plasmids is thus proposed to be the product of the *N. capsulatus* (Bath) *glnA* gene.

4:2:4 Determination of the limits and direction of transcription of the cloned *N. capsulatus* (Bath) *glnA* gene.

In order to determine the approximate limits and direction of transcription of the cloned *N. capsulatus* (Bath) *glnA* gene, various restriction endonuclease digests of the recombinant pDC2 were made. (The plasmid pDC2 was chosen for these determinations for two reasons; i) Synthesis of the M_r 60,000 polypeptide (*glnA* gene product) is greater relative to the same polypeptide produced by pDC1; ii) the origin of the M_r 42,000 polypeptide encoded solely by pDC2 may be derived by these studies. Each restriction endonuclease digest of pDC2 was carried out to completion, phenol/chloroform, then chloroform extracted and finally ethanol precipitated. 5 μg of each restricted pDC2 DNA were then used as templates

Table 4:2 Pattern of *in vivo* polypeptide synthesis by pDC1 and derivatives.

Plasmid	M_r of Polypeptides			
	60,000 *glnA*	42,000	30,000 *bla*	26,000 *cat*
pDC1	++	-	++	-
pDC2	+++	+++	++	-
pDC100	+++	-	++	-
pBR325	-	-	++	+++

The relative proportions of different polypeptides synthesized is indicated on a scale ++++ to + as judged by visual inspection.

- = not detected.

for the *in vitro* coupled transcription-translation system outlined previously, with ^{35}S-methionine as the labelled amino acid. The resulting polypeptides produced were electrophoresed on a 10% SDS denaturing polyacrylamide gel and visualized by fluorography. The resulting fluorograph (Figure 4:4) clearly shows that when either *Kpn*I, *Sal*I or *Hin*cII restricted pDC2 DNA were used as the template in this system, the loss of the M_r 60,000 polypeptide was observed. However, if *Cla*I or *Nco*I restricted pDC2 DNA was used as the template, the M_r 60,000 polypeptide was still synthesized. *Kpn*I restricted pDC2 DNA did not yield any discernible truncated polypeptides in this system. However, when *Sal*I and *Hin*cII restricted pDC2 DNA's were used as templates in this system, each yielded discernible truncated polypeptides (see Figure 4:4). (N.B. *Hin*cII also cleaves within the pBR325 encoded β-lactamase (*bla*) gene, therefore loss of the *bla* gene product is observed). This data suggests that the *Kpn*I site in pDC2 is located within, or is just downstream of the regulatory region for the *N. capsulatus* (Bath) *gln*A gene encoding the M_r 60,000 polypeptide.

This data, together with the heterologous hybridization data (see Chapter 3), the complementation analysis data of pDC1 and its derivatives and the fact that expression of the M_r 60,000 polypeptide is much greater from pDC2 relative to pDC1, indicate that the direction of transcription occurs from left to right in pDC2 (i.e. the same relative orientation as the chloramphenicol acetyl transferase gene of pBR325) or right to left in pDC1 (i.e. opposing the chloramphenicol acetyl transferase gene of pBR325) (see Figure 4:5).

The origin of the M_r 42,000 polypeptide encoded by pDC2 remains to be elucidated, as none of the restriction endonucleases used in cleaving the pDC2 DNA template resulted in the loss of this polypeptide. When *Eco*RI restricted pDC2 DNA was used as a template in the *in vitro* coupled transcription-translation system (see Figure 4:6), the M_r 60,000 polypeptide was still synthesized (firm evidence as to the existence of its

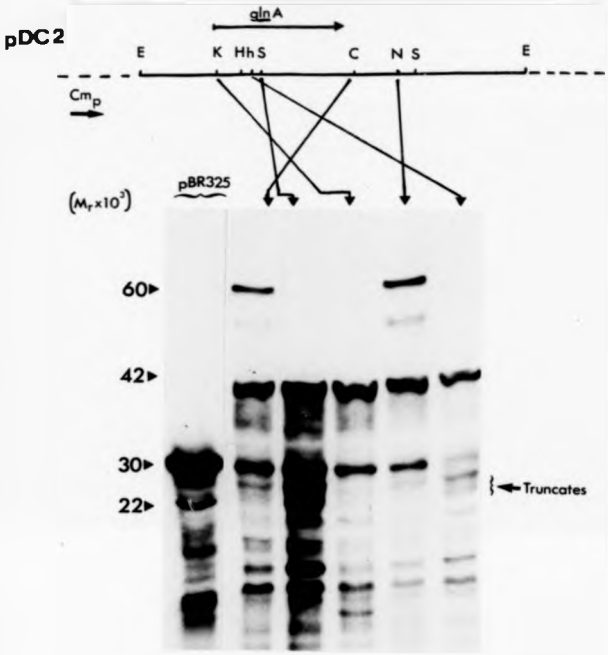

pDC2
glnA
E
K
Hh
S
C
N
S
E
Cm
p
pBR325
(Mr x10³)
60
42
30
22
Truncates

Figure 4:4 *In vitro* synthesized polypeptide products of endonuclease restricted pDC2 templates.

The fluorograph shows the ^{35}S-methionine labelled polypeptide products of various endonuclease restricted pDC2 templates. The M_r 30,000 and 22,000 polypeptides are the β-lactamase and chloramphenicol acetyl transferase products of pAR325.

Key:

E - *Eco*RI

K - *Kpn*I

h - *Hinc*II

H - *Hind*III

S - *Sal*I

C - *Cla*I

N - *Nco*I

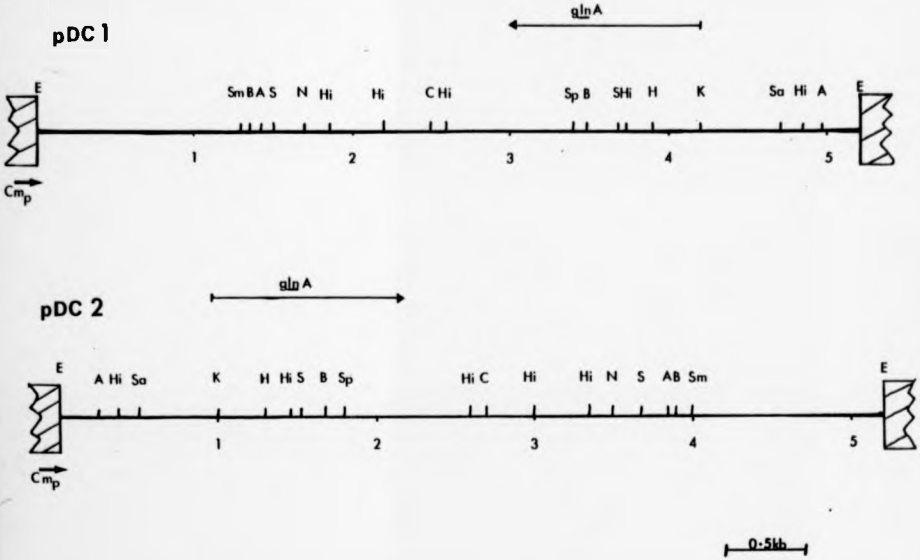

pDC 1
glnA
E
Sm B A S N Hi Hi C Hi Sp B SHi H K Sa Hi A
E
1
2
3
4
5
Cmp
pDC 2
glnA
E
A Hi Sa K H Hi S B Sp Hi C Hi Hi N S AB Sm
E
1
2
3
4
5
Cmp
0·5kb

Figure 4.5 Endonuclease restriction maps of pDC1 and pDC2 inserts indicating limits and direction of transcription of *glnA*.

Key:

E = *EcoR*I
K = *Kpn*I
H = *Hind*III
Hi = *Hinc*II
S = *Sal*I
Sa = *Sac*I
Sp = *Sph*I
B = *Bam*HI
C = *Cla*I
N = *Nco*I
A = *Ava*I
Sm = *Sma*I

Cmp = chloramphenicol acetyl transferase promoter-direction of transcription.

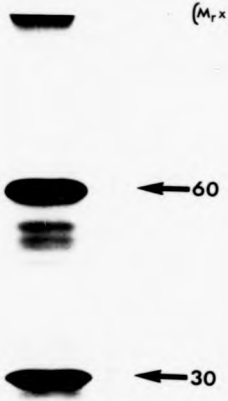

($M_r \times 10^3$)
60
30

Figure 4.6 *In vitro* translation products of an *Eco*RI restricted pDC2 template.

The fluorograph shows the products of an *Eco*RI restricted pDC2 DNA template. The M_r 30,000 polypeptide corresponds to β-lactamase of pBR325.

own regulatory region) whereas the M_r 42,000 polypeptide is no longer synthesized. This result suggests that the origin of the coding region for this polypeptide is within the cloned 5.2 Kb *Eco*RI insert of pDC2, downstream of *glnA* beyond the *Sal*I site (Figure 4:6-map coordinate 3.85 Kb on the pDC2 map) and reading through into the C-terminal coding region of the chloramphenicol acetyl transferase gene of pBR325, producing a fusion product. The origin of this protein is known not to be upstream of *glnA* in pDC2 i.e. from the chloramphenicol acetyl transferase constitutive promoter of pBR325, as the existance of the *Hin*cII site (map coordinates 0.35 Kb on the pDC2 map - Figure 4:5), does not result in the loss of the M_r 42,000 polypeptide upon subsequent expression of a *Hin*cII restricted pDC2 template, in the coupled transcription-translation system (see Figure 4:4).

The complete nucleotide sequence of the *N. capsulatus* (Bath) structural gene, together with upstream and downstream sequences are presented in Chapter 5 of this thesis, providing conclusive evidence as to the limits and direction of transcription of the *N. capsulatus* (Bath) *glnA* gene within pDC1/2.

4:2:5 Mutagenesis of the cloned *N. capsulatus* (Bath) *glnA* region.

In order to determine the location and minimum size of the cloned *glnA* gene and at the same time produce a mutant allele with a selectable marker for marker exchange experiments, attempts were made to mutagenize plasmid pDC1 with the transposon Tn5, and the omega (Ω) fragment of Prentki and Krisch (Prentki and Krisch, 1984).

4:2:5:1 Tn5 mutagenesis.

Transposons are specialised DNA elements which move to new genetic locations without extensive sequence homology. Transposon Tn5 confers kanamycin resistance (Berg *et al.*, 1975) and genetic mapping has indicated that Tn5 can insert into many sites within a single gene and also at many chromosomal locations (Berg, 1977; Shaw and Berg, 1979). When Tn5 transposes into a structural gene, it abolishes gene function through insertional inactivation and exerts a polar effect on genes within an operon distal to its insertion (Berg *et al.*, 1980). Transposon Tn5 was chosen for these studies as it confers kanamycin resistance, kanamycin being one of the few antibiotics which has been found to be stable at 45°C (the optimum growth temperature of *N. capsulatus* (Bath)) and was therefore an important consideration for future marker exchange studies (see Chapter 7).

E. coli CSH26ΔF6 cells (*sup*°) harbouring pDC1 were infected with λ_{467} (see Chapter 2, Section 2:17) and plated onto LB agar containing ampicillin, tetracycline (for plasmid selection) and kanamycin (300 μg ml^{-1} for Tn5 selection). Plasmid DNA was prepared from all the $Ap^{R}Tc^{R}Km^{R}$ transductants (approximately 2,500) and used to transform *E. coli* ET8894 (*glnA-ntrC*)∇ cells. Transformants were screened for their GlnA phenotype and of 300 screened none were found to be $GlnA^{-}$. In order to determine whether any Tn5 insertions had occurred within the 5.2 kb *N. capsulatus* (Bath) *Eco*RI insert of pDC1, plasmid DNA was prepared from 70 $Ap^{R}Tc^{R}Km^{R}$ ET8894 transformants (ten transformants per plasmid preparation), digested to completion with *Eco*RI (which results in the release of the pDC1 insert from vector DNA, but does not cleave Tn5) and samples electrophoresed on a 0.5% (v/v) agarose gel. The resulting gel (Figure 4:7) shows that all Tn5 insertions had occurred within vector sequences and that no insertions had occurred within the 5.2 kb *N. capsulatus* (Bath)

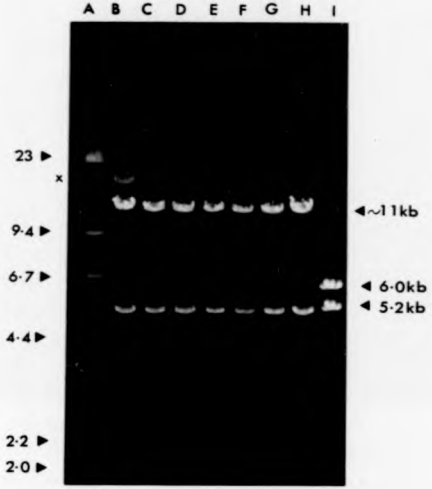
Figure 4:7 Tn5 mutagenesis of pDC1.

Plasmid DNA was isolated from 70 pDC1 :: Tn5 transformants, restricted with *Eco*RI and electrophoresed through a 0.5% (w/v) agarose gel.

Key to tracks:

A	λ DNA restricted with *Hind*III
B	pDC1 :: Tn5 restricted with *Eco*RI from transformants 1-10
C	pDC1 :: Tn5 restricted with *Eco*RI from transformants 11-20
D	pDC1 :: Tn5 restricted with *Eco*RI from transformants 21-30
E	pDC1 :: Tn5 restricted with *Eco*RI from transformants 31-40
F	pDC1 :: Tn5 restricted with *Eco*RI from transformants 41-50
G	pDC1 :: Tn5 restricted with *Eco*RI from transformants 51-60
H	pDC1 :: Tn5 restricted with *Eco*RI from transformants 61-70
I	pDC1 DNA restricted with *Eco*RI

(x = fragment result of partial digestion).


A B C D E F G H I
23
x
9·4
6·7
4·4
2·2
2·0
~11kb
6·0kb
5·2kb

*Eco*RI insert of pDC1. This procedure was repeated on a number of occasions with the same result.

4:2:5:2 Site-directed mutagenesis.

Due to the failure to isolate any Tn5 insertions within the cloned *N. capsulatus* (Bath) *gln*A gene using λ_{esp} as a transposon delivery vehicle, a site-directed mutagenesis approach was undertaken in order to isolate a selectable mutant *N. capsulatus* (Bath) *gln*A allele for marker exchange experiments.

The plasmid pHP45Ω (Prentki and Krisch, 1984) contains the omega (Ω) element which encodes streptomycin (Sm^R) and spectinomycin (Sp^R) resistance. This selectable 2 kb DNA fragment was chosen for these studies as streptomycin is stable at 45°C (see Chapter 7) and therefore can eventually be used for selection of *N. capsulatus* (Bath) *gln*A mutants in marker exchange experiments.

In vitro construction of pDC2/Ω.

The plasmid pHP45Ω was digested to completion with *Hin*dIII to release the 2 Kb Ω fragment. After subsequent purification, the Ω fragment was ligated to phosphatased, *Hin*dIII partially restricted (linearised) pDC2. (The *Hin*dIII site within the pDC2 insert was chosen as this had previously been predicted to lie within the *gln*A open reading frame (see Sections 4:2:4)). The resulting ligation mixture was used to transform competent *E. coli* ET8894 cells and plated onto LB agar containing glutamine (500 $\mu g\ ml^{-1}$), ampicillin, tetracycline and streptomycin. The resulting 50 $Ap^R Tc^R Sm^R$ transformants were replica plated onto M9 glucose minimal agar containing 0.1% (w/v) NH_4Cl, in the presence and absence of glutamine (500 $\mu g\ ml^{-1}$), in order to determine their GlnA phenotype. All 50 transformants were

subsequently found to be GlnA$^-$. (This result was predicted since, although partial digestion with *Hin*dIII can linearize pDC2 from one of two *Hin*dIII sites, selection of transformants on tetracycline containing media discriminates against those recombinants in which the Ω fragment has inserted into the vector *Hin*dIII site, thereby leading to tetracycline sensitivity). Plasmid DNA from a single GlnA$^-$ transformant was isolated and subsequently digested with *Sal*I to verify correct insertion of the Ω fragment into pDC2 (data not presented).

4:2:6 Enzyme activities of the cloned *N. capsulatus* (Bath) *glnA* gene product in *E. coli glnA* (*ntr*$^+$ and *ntr*$^-$) strains.

Glutamine synthetase activity was assayed by the Mn^{2+}-dependent-γ-glutamyl transferase assay in cell-free extracts obtained from exponential-phase *E. coli* TH16 (*glnA ntr*$^+$), ET8894 (*glnA ntrB* ntrC) and ET8000 (*glnA*$^+$ *ntr*$^+$) cells, as well as TH16 and ET8894 harbouring pDC1 or one of its derivatives. GS specific activity could not be detected in either TH16 or ET8894, but significant levels were detected in ET8000 and strains harbouring pDC1, pDC2 or pDC100 (see Table 4:3). GS assays were carried out at 37°C (*E. coli* optimum growth temperature) and at 45°C (*N. capsulatus* (Bath) optimum growth temperature).

The effect of nitrogen starvation on the expression of the cloned *N. capsulatus* (Bath) GS activity in *E. coli* was determined by comparing GS activity from cells grown under nitrogen excess (15 mM glutamine) or nitrogen-limiting conditions (0.15 mM glutamine) (Table 4:3). GS activity in ET8000 was repressed by excess nitrogen and was induced (9-fold increase) under conditions of limiting nitrogen. Although the cloned *N. capsulatus (Bath) glnA* gene in TH16 produced high levels of GS activity in excess nitrogen (possibly due to plasmid copy number effect), pDC1-encoded GS activity was increased approximately sixteen-fold when cells were

Table 4.3 Activity of *M. capsulatus* (Bath) glutamine synthetase in *E. coli* Ntr$^+$ and Ntr$^-$ strains.

Strain	Growth on 0.2% arginine as sole nitrogen-source	GS activity[b] in concentration of glutamine[a] (mM)				Phenotype
		0.15		15		
		37°C	45°C	37°C	45°C	
ET8000	+	1.80	0.26	0.20	0.15	GlnA$^+$ Ntr$^+$
TH16	-	ND	ND	ND	ND	GlnA$^-$ Ntr$^+$
TH16/pDC1	+	12.24	14.28	0.77	0.89	GlnA$^+$ Ntr$^+$
TH16/pDC2	+	80.52	109.10	71.43	114.29	GlnA$^+$ Ntr$^+$
TH16/pDC100	+	18.17	21.09	0.94	1.62	GlnA$^+$ Ntr$^+$
ET8894	-	ND	ND	ND	ND	GlnA$^-$ Ntr$^-$
ET8894/pDC1	-	0.34	0.43	0.36	0.49	GlnA$^+$ Ntr$^-$
ET8894/pDC2	-	97.60	111.90	94.21	102.30	GlnA$^+$ Ntr$^-$
ET8894/pDC100	-	0.35	0.41	0.30	0.38	GlnA$^+$ Ntr$^-$

All assay results are the product of the mean of three separate determinations.

(a) Growth medium was glucose M9 minimal medium containing 15 mM glutamate plus the concentration of glutamine indicated.

(b) GS specific activity expressed as μmoles γ-glutamyl hydroxamate min^{-1} mg^{-1} protein.

ND = Not detected

\+ = Growth

\- = No growth

shifted to limiting nitrogen conditions and high levels of GS activity were obtained. GS specific activity of cell extracts prepared from TH16 harbouring pDC100 appeared to parallel that of TH16 harbouring pDC1 i.e. regulated by nitrogen levels.

pDC2 encoded GS activity on the otherhand, was extremely high, in either strain background, both under nitrogen-limiting and nitrogen-excess conditions. GS specific activity in the cells complemented by the *N. capsulatus* (Bath) *glnA* gene encoded by pDC2 may be due to unregulated overexpression of this gene, caused by readthrough from the constitutive chloramphenicol acetyl transferase promoter in the vector pBR325. This overexpression of the pDC2 encoded *glnA* gene product was also observed by the greater relative intensity of the pDC2 encoded M_r 60,000 polypeptide product, compared to the pDC1 encoded M_r 60,000 polypeptide from the *in vitro* transcription-translation studies (see Subsection 4:2:3:1). The relative orientation of the cloned *glnA* gene with respect to the chloramphenicol acetyl transferase promoter of pBR325 in pDC1 and pDC2 also lends support to this theory (Subsection 4:2:4).

The *E. coli* (*glnA-ntrC*) deletion mutant ET8894 harbouring either pDC1 or pDC100 produced low levels of GS specific activity irrespective of the nitrogen-status.

The effect of temperature on GS specific activity as measured by the γ-glutamyl transferase assay was determined. GS specific activity encoded by the recombinants pDC1, pDC2 and pDC100 was higher at 45°C whereas the chromosomally encoded GS of ET8000 showed higher activity at 37°C. These results parallel the optimum growth temperatures of the organisms from which the GS structural gene originated.

The activity of the cloned *N. capsulatus* (Bath) *glnA* gene product was demonstrated in 5% (w/v) non-denaturing polyacrylamide tube gels and 4%-15% non-denaturing slab gels using the γ-glutamyl transferase activity stain. Tube gels were loaded with 50 μg of cell-free extract prepared from *E. coli* TH16 and TH16 harbouring pDC1 grown on glucose minimal medium containing either limiting nitrogen (0.15 mM glutamine) or excess nitrogen (15 mM glutamine). Following electrophoresis, one set of tube gels was subjected to the γ-glutamyl transferase activity stain and one set stained with Coomassie blue (Figure 4:8). Slab gels were loaded with 100 μg of cell-free extract prepared from *E. coli* TH16, TH16/pDC1 (grown on glucose minimal medium containing 0.1% (w/v) NH_4Cl), and also *N. capsulatus* (Bath) grown in MS, NMS and AMS media (see Figure 4:9). The characteristic brown band of γ-glutamyl hydroxamate can be seen in both tube and slab gels after activity staining. The GS enzyme encoded by pDC1 can be seen to have the same relative mobility within the 4%-15% polyacrylamide slab gel as the *N. capsulatus* (Bath) GS enzyme in the MS, NMS and AMS prepared cell-free extracts (see Figure 4:9).

In *E. coli*, the products of the *ntrB* and *ntrC* genes which are closely linked to the *glnA* gene, together with the *ntrA* gene product, regulate the expression of five genes in the arginine degradation pathway (Shaibe *et al.*, 1985). The ability of the *E. coli* strains ET8000, TH16, ET8894 together with, TH16 and ET8894 harbouring pDC1, pDC2 and pDC100 to grow on minimal medium containing arginine as sole nitrogen source, was determined (Table 4:3) in order to ascertain the presence of analogous *ntr* genes on these recombinants. The recombinants pDC1, pDC2 and pDC100 allowed the Ntr^+ strain TH16 to grow on this medium by complementing the *glnA* lesion in this organism. The Ntr^- strain, ET8894, was not complemented by the recombinants pDC1, pDC2 or pDC100 for growth on arginine as sole nitrogen source.

Figure 4.8 Non-denaturing polyacrylamide tube gels (5% w/v acrylamide) demonstrating activity stain for glutamine synthetase.

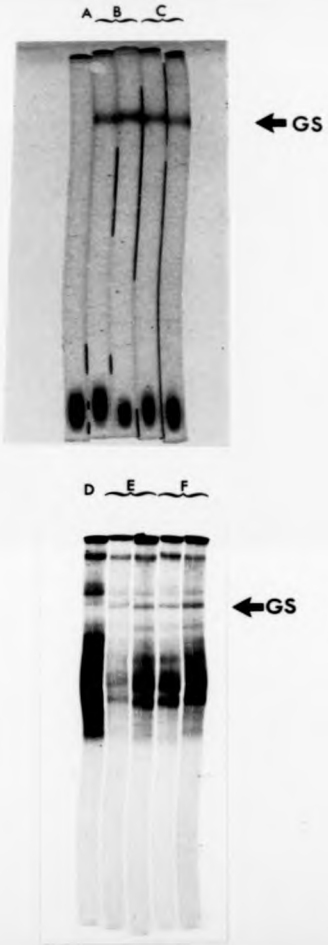
Key to gels:

A 50 μg TH16 cell extract γ-glutamyl transferase activity stained

B 50 μg TH16/pDC1 cell extract prepared from cells grown in limiting nitrogen (0.15 mM glutamine) γ-glutamyl transferase activity stained.

C 50 μg TH16/pDC1 cell extract prepared from cells grown in excess nitrogen (15 mM glutamine) γ-glutamyl transferase activity stained.

Gels D to F correspond to gels A to C stained with Coomassie Blue.


A
B
C
GS
D
E
F
GS

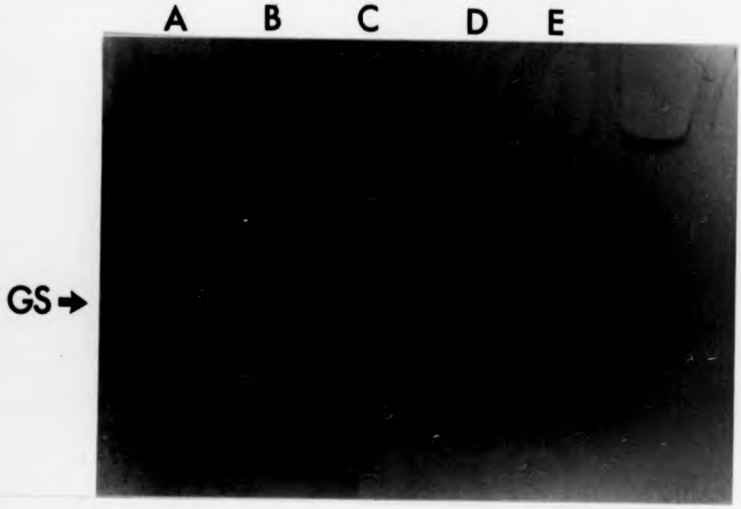

A
B
C
D
E
GS

Figure 4:2 Non-denaturing polyacrylamide gradient slab gel demonstrating activity stain for glutamine synthetase (GS).

100 μg of each cell-free extract were electrophoresed through a 4-15% (w/v) polyacrylamide gradient gel and GS visualized with the γ-glutamyl transferase activity stain.

Key:

A TH16/pDC1 cell-free extract

B TH16 cell-free extract

C *M. capsulatus* (Bath) cell-free extract prepared from cells grown on AMS

D *M. capsulatus* (Bath) cell-free extract prepared from cells grown on NMS

E *M. capsulatus* (Bath) cell-free extract prepared from cells grown on MS

4:3 Discussion.

The 5.2 Kb DNA fragment cloned from *M. capsulatus* (Bath) was shown to complement the *glnA* lesions present in *E. coli* TH16 and KT8894, as well as in *K. pneumoniae* UNF1848. These results show that the *Methylococcus* gene can function both in *E. coli* and *K. pneumoniae* as well as to share DNA sequence homology with the *K. pneumoniae glnA* gene as determined by hybridization analysis (see Chapter 3).

In vitro transcription and translation studies together with the *in vivo* 'maxi' cell studies, with plasmid DNA containing the functional *M. capsulatus* (Bath) *glnA* gene and portions of the 5.2 Kb *M. capsulatus* (Bath) DNA insert, indicated that the M_r 60,000 polypeptide was the *M. capsulatus* (Bath) GS subunit. The size of the *M. capsulatus* (Bath) GS subunit determined in these studies is in agreement with the previously reported *M. capsulatus* (Bath) GS monomer (Murrell and Dalton, 1983(c)). The M_r 60,000 polypeptide was also synthesized by pDC2, which carries the same *Eco*RI fragment of pDC1 but in the reverse orientation. In addition, pDC2 directed the synthesis of a M_r 42,000 polypeptide, which appears to be the result of a fusion between part of the chloramphenicol acetyl transferase gene of pBR325 and a gene carried in the cloned fragment.

In vitro transcription and translation studies together with hybridization and complementation studies, revealed the direction of transcription of the cloned *glnA* gene within the cloned 5.2 Kb fragment, to be opposing the chloramphenicol acetyl transferase promoter of pBR325 in pDC1 and in the same relative orientation of this promoter in pDC2. This factor is reflected both in the amounts of the M_r 60,000 polypeptide synthesized in both *in vitro* and *in vivo* expression systems, as well as in the GS assay results.

Mutagenesis studies with the transposable genetic element Tn5 yielded no insertions within the cloned 5.2 Kb *N. capsulatus* DNA fragment. The reason for this phenomenon is at present unknown. However, recent studies using pBR322 as a target for Tn5 have revealed a complex distribution of frequently and infrequently used sites. Tn5 was found to have two 'hotspots' of insertion within the *tet* locus (Berg *et al.*, 1988). This suggests that the pBR325 based recombinant pDC1, may also contain Tn5 'hotspots', however, any insertions within the β-lactamase or tetracycline resistance genes can be discounted, as initial selection for transposition of Tn5 was made in the presence of both ampicillin and tetracycline. Previous studies using pBR325 based recombinants as targets for Tn5 have not reported similar findings e.g. Toukdarian and Kennedy, 1986. These findings therefore, suggest that there may be some factor pertaining to the cloned *N. capsulatus* (Bath) DNA fragment within pDC1, which prevents Tn5 insertion. Previous studies have shown that the topology of the target DNA is important for Tn5 transposition and that insertion specificity may depend on local DNA conformation (Berg *et al.*, 1988). Further studies are therefore required in order to elucidate this phenomenon occurring with pDC1.

The *N. capsulatus* (Bath) *glnA* gene in *E. coli* produced relatively high levels of GS activity in excess nitrogen. Nevertheless, the *N. capsulatus* (Bath) *glnA* gene was subject to nitrogen regulation in Ntr$^+$ *E. coli* (TH16) (when the recombinants pDC1 or pDC100 directed synthesis), as under conditions of limiting nitrogen, there was an approximate sixteen fold increase in GS activity and very high levels of GS activity were produced. When the recombinants pDC1 or pDC100 were placed in an Ntr$^-$ *E. coli* (ET8894), unregulated, low levels of GS specific activity were observed, irrespective of the nitrogen status. This unregulated, low level of GS activity in this strain background may be due to the presence of two promoters upstream of *glnA* (see Chapter 5), as found in at least three

genera of the family Enterobacteriaceae, in which the upstream promoter (*glnAp1*) is negatively regulated and the downstream promoter (*glnAp2*) is positively regulated, by the products of the *ntr* system (Dixon, 1984; Reitzer and Magasanik, 1985). Under nitrogen-limiting conditions, NtrC-P both stimulates transcription from *glnAp2* and inhibits expression from *glnAp1*. Under nitrogen-excess conditions, NtrC-P is dephosphorylated, which in turn relieves repression of transcription from *glnAp1* and leads to a loss in *glnAp2* expression. Mutations in *ntrB* or *ntrC* therefore, can lead to low level unregulated expression of *glnA* from *glnAp1* and an Ntr^- phenotype, as well as glutamine auxotrophy (McFarland *et al.*, 1981; Wei and Kustu, 1981). This phenomenon has been observed with a *S. typhimurium* (*glnA-glnB*) deletion strain harbouring the *E. coli glnA* gene (McFarland *et al.*, 1981). Unregulated, low level expression of the *H. capsulatus* (Bath) *glnA* gene in ET8894 (from both pDC1 and pDC100) together with the inability to complement the Ntr^- phenotype of this strain, indicates that there is no functional regulatory region downstream of *glnA* in these recombinants, and that regulation of *H. capsulatus* (Bath) *glnA* in TH1ℓ is due to a functional *E. coli ntr* system.

In contrast, the structural gene for GSII from *Rhizobium leguminosarum* has been shown to complement an $ntrC^+$ but not an *ntrC K. pneumoniae glnA* mutant (Filser *et al.*, 1986). This observation suggests that a functional *ntrC* gene product is required *per se* for expression of the GSII structural gene from this organism. The structural gene for GSII (*glnII*) has also been cloned and sequenced from *Bradyrhizobium japonicum* (Carlson and Chelm, 1986). This gene is expressed in an *E. coli glnA-ntrC* deletion strain (ET8051), only when the gene is expressed from the *tet* promoter of the vector, pRR322. A subsequent report revealed the existence of a regulatory region on the cloned fragment containing *glnII* of *B. japonicum*. This regulatory region consists of a single Ntr-consensus type promoter and an NtrC binding site (Carlson *et al.*, 1987). Using a quantitative nuclease

protection procedure, expression of *glnII* was shown to be induced when growth was limited by nitrogen source depletion i.e. under *ntr* control.

The expression of the cloned *M. capsulatus* (Bath) GS activity encoded by pDC1 and pDC100 has been shown by these studies to be regulated by nitrogen levels in *E. coli*, in an Ntr^+ but not in an Ntr^- background. This suggests that a similar or analogous Ntr system may be present in *M. capsulatus* (Bath). Previous studies with *M. capsulatus* (Bath) by Murrell and Dalton (1983(b)) showed that GS specific activity as measured by the γ-glutamyl transferase assay, was repressed in nitrogen-excess conditions but increased six-fold under nitrogen-limiting conditions. GS biosynthetic activity also increased dramatically under nitrogen-limiting conditions. The increase in the amount of total GS protein under nitrogen-limiting conditions must therefore be regulated at the level of expression, possibly by a system analogous to the Ntr system of the Enterobacteriaceae (reviewed in Merrick, 1988(a)). This theory is borne out by the result obtained from expression studies carried out on the *M. capsulatus* (Bath) cloned *glnA* gene encoded by pDC1 in *E. coli*, both in Ntr^+ and Ntr^- backgrounds. Evidence is presented in Chapters 5 and 6 of this thesis, indicating the presence of an analogous Ntr system. However, the genetic arrangement of the *ntr*-like genes is markedly different to that found in the Enterobacteriaceae.

An analogous Ntr system to that in the Enterobacteriaceae has also been detected in *Azotobacter vinelandii* (Toukdarian and Kennedy, 1986; Kennedy and Toukdarian, 1987), including the conservation of the *glnA-ntrB-ntrC* linkage. However, unlike that found in the enterics, expression of *glnA* in *A. vinelandii* is not under *ntr* regulation (Santero *et al.*, 1986; Toukdarian and Kennedy, 1986). It had been demonstrated previously that the amount of GS protein in *A. vinelandii* did not vary significantly with the nitrogen status of the cell (Kleinschmidt and Kleiner, 1981; Lepo *et al.*, 1982), possibly due to the assimilation of ammonia in this organism proceeding exclusively via GS and GOGAT. This phenomenon is also reflected

in the Type II methanotrophs, which assimilate ammonia exclusively via GS/GOGAT but show no variance in the amount of GS protein in cells grown under nitrogen limiting or nitrogen excess conditions. Type I methanotrophs on the other hand, which assimilate ammonia via GDH or GS/GOGAT depending on the nitrogen source, show a great variance in total GS protein in cells grown under conditions of nitrogen limitation or excess (Murrell and Dalton, 1983(b)). It is interesting to speculate therefore, that in Type II methanotrophs, if an Ntr-like system is present (i.e. possibly for nitrogen fixation capability) it is unlikely to regulate GS synthesis. Conversely, expression of GS in Type I methanotrophs is likely to be under Ntr-regulation. Some evidence as to the presence of an Ntr system in the obligate methanotrophs will be presented in Chapter 6 of this thesis.

Chapter 5

Nucleotide sequence of the *M. capsulatus* (Bath) glutamine synthetase structural gene.

5:1 Introduction.

To date there is no information available on the molecular structure of genetic material from *M. capsulatus* (Bath) or any other obligate methanotroph. It has been shown from the studies carried out for this thesis that, methanotroph DNA can be expressed and produce a functional protein product in a heterologous host. To complement the expression studies, the complete nucleotide sequence of the *M. capsulatus* (Bath) *glnA* structural gene was determined, to enable comparative analyses to be made between the *M. capsulatus* (Bath) *glnA* gene and *glnA* genes from a number of other organisms, with respect to promoter structure, preferred codon usage and derived amino acid sequences.

5:2 Results.

5:2:1 Nucleotide sequence of the *M. capsulatus* (Bath) *glnA* gene.

The physical map of the *M. capsulatus* (Bath) *glnA* region previously determined (see Chapter 4), was used as a basis for the sequencing strategy outlined in Figure 5:1. The specific restriction fragments required for sequencing were all derived from the plasmid pDC100. Each specific restriction fragment (as outlined in Figure 5:1) was prepared by digestion of pDC100 with the appropriate restriction enzymes, fractionated on a 2% preparative agarose gel (as described in Materials and methods, Chapter 2) and subcloned in both possible orientations into bacteriophage M13 strains

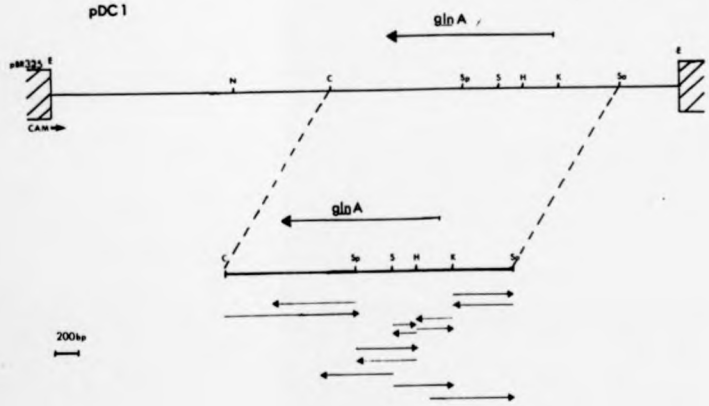

pDC1
glnA
pBR325
E
N
C
Sp
S
H
K
Sa
E
CAM
glnA
C
Sp
S
H
K
Sa
200bp

Figure 5:1 Sequencing strategy for the *N. capsulatus* (Bath) *glnA* gene region.

Key:

E	*EcoR*I
K	*Kpn*I
Sa	*Sac*I
H	*Hind*III
S	*Sal*I
Sp	*Sph*I
C	*Cla*I
N	*Nco*I

tgl30 and tgl31. *E. coli* strain TG1 was used as host for the M13 tg phages. Each restriction fragment was sequenced by the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977).

N. capsulatus (Bath) DNA (2,463 bp), which contained the *glnA* gene was sequenced (Figure 5:2). The DNA sequence contained two putative open reading frames (ORFs) of 1407 bp and 197 bp (nucleotide positions 503 to 1909 and 2010 to 2207 respectively). In addition the 3' terminus of a third putative ORF is located at nucleotide positions 1 to 334. The larger ORF was preceded by a consensus Shine-Dalgarno (SD) sequence (AGGAGGA) (Shine and Dalgarno, 1976) 10 bp upstream from the presumptive start codon (ATG). This ORF encoded a polypeptide of 468 amino acid residues with a predicted M_r of 51,717. The smaller ORF lies directly downstream of the large ORF and is preceded by a SD sequence (AAAGGAAG) 11 bp upstream from the presumptive start codon (ATG). This ORF encodes a polypeptide of 65 amino acid residues with a predicted M_r of 7022. The *N. capsulatus* (Bath) GS monomer is encoded by the large (1407 bp) ORF.

5:2:2 The *N. capsulatus* (Bath) *glnA* gene transcription control region.

In the Enterobacteriaceae, transcription of the *glnA* gene is from one of two functional promoters (Dixon, 1984; Reitzer and Magasanik, 1985; Kustu *et al.*, 1986). The upstream promoter (*glnAp1*) resembles the canonical -35 and -10 promoter whilst the downstream promoter (*glnAp2*) resembles the NtrA-dependent promoters with conserved residues at -24 and -12 with respect to the transcription start site.

In the *N. capsulatus* (Bath) *glnA* gene leader region both types of promoter element were detected (Figure 5:3). Two putative *glnAp1*-type promoters (P_1 and P_3) were found located either side of a *glnAp2*-type promoter element (P_2) in the *N. capsulatus* (Bath) *glnA* leader region. The nucleotide sequences of the three types of putative *N. capsulatus* (Bath) *glnA* promoters are given below:-

Figure 5:2 Nucleotide sequence of the *N. capsulatus* (Bath) *glnA* region.

The sequence consists of 2,463 bp containing two open reading frames (ORFs) of 1407 bp and 197 bp and also the 3' terminus of a third putative ORF - displayed in bold print.

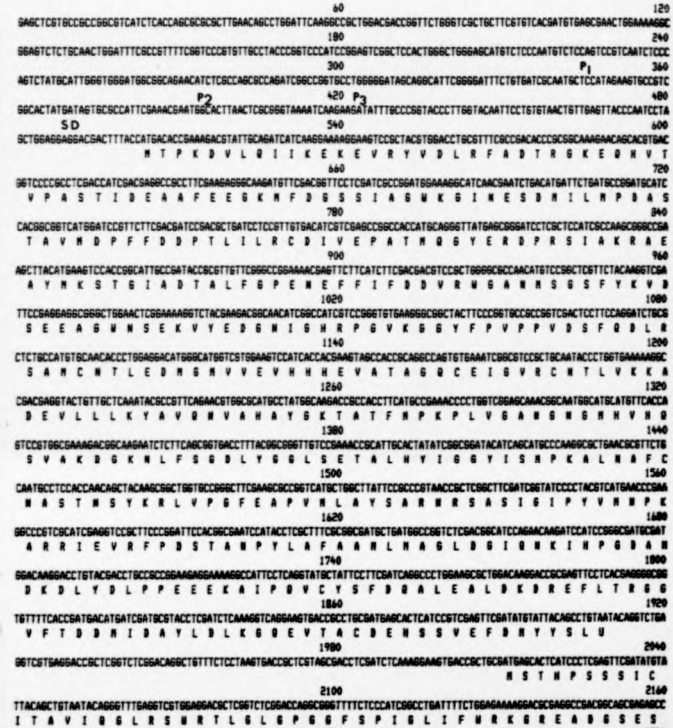

P2
P3
P1
SD

Figure 5.3 The nucleotide sequence of the *N. capsulatus* (Bath) *glnA* transcription control region.

The three presumptive promoter elements are displayed in bold type. The putative NtrC binding site is boxed.

```
                                                                    40
GAGCTCGTGCCGCCGGCGTCATCTCACCAGCGCGCGCTTGAACAGCCTGGATTCAAGGCC

                                                                   120
GCTGGACGACCGGTTCTGGGTCGCTGCTTCGTGTCACGATGTGAGCGAACTGGAAAAGGC

                                                                   180
GGAGTCTCTGCAACTGGATTTCGCCGTTTTCGGTCCCGTGTTGCCTACCCGGTCCCATCC

                                                                   240
GGAGTCGGCTCCACTGGGCTGGGAGCATGTCTCCCAATGTCTCCAGTCCGTCAATCTCCC

                                                                   300
AGTCTATGCATTGGGTGGGATGGCGGCAGAACATCTCGCCAGCGCCAGATCGGCCGGTGC

                                                        P1         360
CTGGGGGATAGCAGGCATTCGGGGATTTCTGTGATCGCAATGCTCCATAGAAGTGCCGTC

                                                   P2              420
GGCACTATGATAGTGCGCCATTCGAAACGAATGGCACTTAACTCGCGGGTAAAATCAAGA

P3                                                                 480
AGATATTTGCCCGGTACCCTTGGTACAATTCCTGTGTAACTGTTGAGTTACCCAATCCTA

   SD
GCTGGAGGAGGACGACTTTACCATG
```

	-35		-10
Consensus canonical promoter	TTGACA	N_{16}	TATAAT
Putative *N. capsulatus* (Bath) *glnA*p1-type promoter (P_1)	GCTCCA	N_{16}	TATGAT
Putative *N. capsulatus* (Bath) *glnA*p1-type promoter (P_2)	AAGATA	N_{16}	TACAAT

	-24		-12
NtrA-dependant promoter consensus sequence	CTGGCAC	N_5	TTGCA
Putative *N. capsulatus* (Bath) *glnA*p2-type promoter (P_3)	ATGGCAC	N_5	TCGCG

The putative *glnA*p1-type promoters (P_1, P_2) are located at nucleotide positions 342 to 371 and 420 to 449 respectively. The putative *glnA*p2-type promoter (P_3) is located at nucleotide positions 391 to 407.

The *N. capsulatus* (Bath) *glnA* gene leader region also contained a single region of dyad symmetry at nucleotide positions 361 to 378, which had a 11 bp out of 15 bp match to the consensus sequence for the binding of the *ntrC* gene product proposed by Dixon (1984) (see below).

	5'		3'
Consensus NtrC binding sequence	TGCACTA	N_5	TGGTGCAA
Putative *N. capsulatus* (Bath) NtrC binding sequence	GGCACTA	N_5	TAGTGCGC

Unlike *E. coli* and *Thiobacillus ferrooxidans* (Reitzer and Magasanik, 1985; Rawlings *et al.*, 1987), no catabolite-activating protein (CAP) consensus recognition sequences were detected in the *N. capsulatus* (Bath) *glnA* gene leader region.

5:2:3 Downstream region of the *N. capsulatus* (Bath) *glnA* gene.

In enteric bacteria, *glnA* mRNA transcription is terminated at a characteristic *rho*-independent terminator comprising of hairpin loop structures (MacFarlane and Merrick, 1985; Krajewska-Grynkiewicz and Kustu, 1984), downstream of the *glnA* stop codon. No regions with inverted symmetry, possessing the potential to form hairpin structures in the resulting mRNA were detected in the 3' flanking region of the *N. capsulatus* (Bath) *glnA* gene. However, a small ORF comprising of 197 nucleotides was detected 100 bp downstream of the *glnA* stop codon. This ORF encodes a polypeptide of 65 amino acid residues with a predicted M_r of 7022. The hydropathy profile of the polypeptide encoded by this ORF is detailed in Figure 5:4. It can be seen that the N-terminal portion of this polypeptide is hydrophobic and the C-terminal portion, mainly hydrophilic. However, the function of this protein remains to be elucidated.

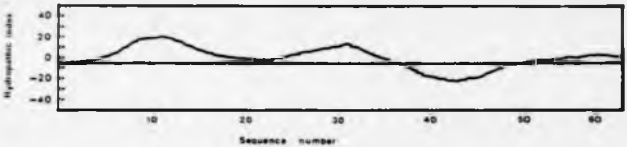
5:2:4 Analysis of the *N. capsulatus* (Bath) *glnA* coding region.

5:2:4:1 Nucleotide sequence analysis.

N. capsulatus (Bath) DNA has a G + C content of 62.5% (Whittenbury and Krieg, 1984), and the *glnA* structural gene exhibits a G + C content of 59%. The codon usage is strongly biased toward the use of codons in which G and C predominate in the third position of all codons (see Figure 5:5).

Figure 5:5 Hydropathic profile of the polypeptide ~~~~~ by the ORF downstream of the *T. cassulatus* (Bath) *glnA* structural gene.

The hydropathic profile shown is based upon the scale of Kyte and Doolittle, (1982). A span of eleven consecutive residues was used.



A comparison between the codon usage patterns of the *glnA* gene from the enteric bacteria *E. coli* and *S. typhimurium* with the non-enteric bacteria *Anabaena* 7120, *Thiobacillus ferrooxidans* and *N. capsulatus* (Bath) is outlined in Table 5:1.

Codon usage in the enteric organisms is essentially the same with the exception of the alanine codons. The preferred codon usage patterns between *N. capsulatus* (Bath) and *T. ferrooxidans* are strikingly similar with the exception of certain proline codons. *T. ferrooxidans* makes frequent use of the proline CCC codon and to a lesser extent the proline CCG codon. *N. capsulatus* (Bath) on the other hand, makes frequent use of the proline CCG codon and rarely uses the proline CCC codon. There are several notable differences in preferred codon usage between the enteric organisms and *N. capsulatus* (Bath). This is apparent in the codons for alanine, lysine and phenylalanine. The overall trend in the preferred codon usage patterns of these bacteria is however similar. The codon usage patterns between *Anabaena* 7120 and the other organisms in this study are substantially different. This is particularly noticeable in the cases of the proline CCG, glycine GGC, arginine CGC and glutamine CAA codons.

A comparison of nucleotide sequences between *N. capsulatus* (Bath) *glnA* and a number of other prokaryotic *glnA* sequences have been determined and are presented in Table 5:2. The *S. typhimurium glnA* gene sequence exhibited the highest degree of homology (70.4%) at the nucleotide level to the *N. capsulatus* (Bath) *glnA* gene, with *C. acetobutylicum* exhibiting the lowest degree of homology (46%). All the *glnA* sequences, with the exception of *C. acetobutylicum*, exhibited greater than 50% homology at the nucleotide level to the *N. capsulatus* (Bath) *glnA* sequence.

Figure 5:5 Codon usage of the *M. capsulatus* (Bath) *glnA* structural gene.

TTT Phe	0 (0.0)	TCT Ser	2 (0.4)	TAT Tyr	6 (1.2)	TGT Cys	2 (0.4)
TTC Phe	22 (4.7)	TCC Ser	14 (3.0)	TAC Tyr	14 (3.0)	TGC Cys	5 (1.1)
TTA Leu	0 (0.0)	TCA Ser	1 (0.2)	TAA End	1 (0.2)	TGA End	0 (0.0)
TTG Leu	5 (1.1)	TCG Ser	6 (1.3)	TAG End	0 (0.0)	TGG Trp	3 (0.6)
CTT Leu	1 (0.2)	CCT Pro	2 (0.4)	CAT His	5 (1.1)	CGT Arg	5 (1.1)
CTC Leu	8 (1.7)	CCC Pro	4 (0.9)	CAC His	6 (1.3)	CGC Arg	10 (2.1)
CTA Leu	0 (0.0)	CCA Pro	1 (0.2)	CAA Gln	0 (0.0)	CGA Arg	0 (0.0)
CTG Leu	17 (3.6)	CCG Pro	18 (3.8)	CAG Gln	11 (2.3)	CGG Arg	3 (0.6)
ATT Ile	2 (0.4)	ACT Thr	0 (0.0)	AAT Asn	5 (1.1)	AGT Ser	0 (0.0)
ATC Ile	19 (4.1)	ACC Thr	14 (3.0)	AAC Asn	13 (2.8)	AGC Ser	4 (0.9)
ATA Ile	0 (0.0)	ACA Thr	1 (0.2)	AAA Lys	8 (1.7)	AGA Arg	0 (0.0)
ATG Met	21 (4.5)	ACG Thr	5 (1.1)	AAG Lys	18 (3.8)	AGG Arg	1 (0.2)
GTT Val	3 (0.6)	GCT Ala	4 (0.9)	GAT Asp	15 (3.2)	GGT Gly	8 (1.7)
GTC Val	16 (3.4)	GCC Ala	23 (4.9)	GAC Asp	23 (4.9)	GGC Gly	24 (5.1)
GTA Val	4 (0.9)	GCA Ala	4 (0.9)	GAA Glu	18 (3.8)	GGA Gly	3 (0.6)
GTG Val	10 (2.1)	GCG Ala	14 (3.0)	GAG Glu	14 (3.0)	GGG Gly	2 (0.4)

Table 5:1

A comparison of the codon usage of the *glnA* gene between *T. ferrooxidans* (Tf), *Anabaena* 7120 (An), *E. coli* (Ec), *S. typhimurium* (Sc) and *M. capsulatus* (Bath) (Mc).

		Tf	An	Ec	Mc	St			Tf	An	Ec	Mc	St
Ala	GCA	3	10	13	4	4	His	CAC	7	7	10	6	12
	GCC	29	7	8	23	14		CAT	8	3	4	5	4
	GCG	9	2	9	14	25	Ile	ATA	0	1	0	0	0
	GCT	2	17	12	4	4		ATC	18	18	16	19	16
Arg	AGA	0	1	0	0	0		ATT	6	13	13	3	11
	AGG	2	0	0	1	0	Leu	CTA	1	6	0	0	0
	CGA	0	0	0	0	0		CTC	8	6	0	8	0
	CGC	10	3	9	10	9		CTG	19	7	29	17	31
	CGG	2	3	0	3	0		CTT	4	2	2	1	0
	CGT	5	8	15	5	13		TTA	0	9	0	0	0
Asn	AAC	10	19	18	13	17		TTG	3	10	1	5	1
	AAT	7	1	2	5	2	Lys	AAA	6	23	19	8	18
Asp	GAC	20	11	19	23	21		AAG	21	8	5	18	7
	GAT	9	17	13	15	10	Met	ATG	16	12	16	21	17
Cys	TGC	4	2	3	5	2	Phe	TTC	16	14	18	22	15
	TGT	0	4	1	2	2		TTT	5	11	5	0	8
Gln	CAA	0	11	2	0	2	Pro	CCA	1	13	6	1	4
	CAG	11	4	9	11	7		CCC	15	6	0	4	1
Glu	GAA	22	24	29	18	28		CCG	7	0	19	18	21
	GAG	10	8	7	14	7		CCT	2	12	4	2	3
Gly	GGA	3	3	1	3	0	Ser	AGC	5	5	1	4	1
	GGC	17	9	19	24	25		AGT	5	2	0	0	0
	GGG	5	0	1	2	2		TCA	0	2	1	1	1
	GGT	13	24	15	8	10		TCC	12	5	13	14	14

		Tf	An	Ec	Mc	St
	TCG	4	0	2	6	0
	TCT	5	14	12	2	9
Thr	ACA	0	13	0	1	0
	ACC	14	9	12	14	16
	ACG	7	2	1	5	2
	ACT	1	3	6	0	3
Trp	TGG	5	8	2	3	2
Tyr	TAC	12	13	12	14	10
	TAT	6	9	5	6	7
Val	GTA	4	6	9	4	4
	GTC	13	3	4	16	9
	GTG	17	1	12	10	11
	GTT	2	11	8	3	7

Table 5:2 Nucleotide sequence comparison between *H. capsulatus* (Bath) *glnA* with published prokaryotic *glnA* sequences.

Organism	Percentage homology to *H. capsulatus* (Bath) glnA	Ref.
Anabaena 7120	59.1	Tumer et al., 1983
Escherichia coli	68.6	Miranda-Rios et al., 1987
Salmonella typhimurium	70.4	Janson et al., 1986
Thiobacillus ferrooxidans	69.9	Rawlings et al., 1987
Clostridium acetobutylicum	46.0	Janssen et al., 1988
Streptomyces coelicolor	63.7	D. Rawlings - pers. comm.
Azospirillum brasilense	69.0	Bozouklian & Elmerich, 1986

5:2:4:2 Nucleotide sequence derived GS amino acid sequence analysis.

1) Prokaryotic.

The nucleotide sequence derived GS amino acid sequences of *E. coli*, *S. typhimurium*, *Anabaena* 7120, *T. ferrooxidans*, *C. acetobutylicum*, *S. coelicolor* and *A. brasilense*, were compared to the nucleotide sequence derived GS amino acid sequence of *H. capsulatus* (Bath) using the IBM AT Microgenie protein alignment subroutine (see Table 5:3). The levels of homology of the DNA derived GS amino acid sequences with the *H. capsulatus* (Bath) GS sequence essentially paralleled the nucleotide homologies, in that *S. typhimurium* exhibited the greatest degree of homology (69%) and *C. acetobutylicum* the lowest (33.2%). This homology increases by greater than 10% if those amino acids which are functionally equivalent are considered (see Table 5:3).

The site of GS adenylylation in *E. coli* has previously been shown to be a tyrosine residue at position 399 in the GS monomer (Heinriksen and Kingdon, 1971). The amino acid sequence around this residue was therefore of interest and each nucleotide derived GS amino acid sequence was compared around this region (see Figure 5:6). The GS enzymes of *E. coli*, *S. typhimurium*, *T. ferrooxidans*, *Anabaena* 7120, *A. brasilense* as well as *H. capsulatus* (Bath) all had a tyrosine residue in this position and showed a strong degree of homology around this residue. *C. acetobutylicum* GS on the other hand, exhibited a low degree of homology in this region (2 out of 12 residues matched the *E. coli* sequence) with a phenylalanine residue in place of the conserved tyrosine residue (Janssen *et al*, 1988).

Another region of interest in the GS monomer is located from residues 270 to 274 in the GS from *E. coli*. This region contains the peptide, MMCMM, which contains an oxidizable histidine residue and is thought to form part of one of the well studied cation-binding sites of GS. Loss of

Table 5.3 Nucleotide sequence derived GS amino acid sequence comparison between *M. capsulatus* (Bath) GS with published prokaryotic GS sequences.

Organism	GS length (aa)	Percentage homology to *M. capsulatus* (Bath) GS	Percentage homology to *M. capsulatus* (Bath) GS including functionally equivalent amino acids	Ref.
Anabaena 7120	474	53.6	66.2	Tumer et al., 1983
E. coli	472	67.6	78.4	Miranda-Rios et al., 1987
S. typhimurium	468	69.0	79.0	Janson et al., 1986
T. ferrooxidans	468	68.3	78.9	Rawlings et al., 1987
C. acetobutylicum	444	33.2	48.5	Janssen et al., 1988
S. coelicolor	469	52.0	65.9	D. Rawlings - pers. comm.
A. brasilense	468	61.9	73.5	Bozouklian & Elmerich, 1986.

Figure 5:6 Comparison of amino acid sequences around the site of GS adenylylation from published prokaryotic sequences.

Organism	Residue	Amino acid sequence	Match	Ref.
		*		
E. coli	394	M D K N L Y D L P P E E	12/12	Miranda-Rios et al., 1987
S. typhimurium	393	M D K N L Y D L P P E E	12/12	Janson et al., 1986
T. ferrooxidans	391	M D K N L Y D L P A E E	11/12	Rawlings et al., 1987
H. capsulatus (Bath)	393	M D K D L Y D L P P E E	11/12	This Work
Anabaena 7120	397	L D K N I Y E L S P E E	8/12	Tumer et al., 1983
A. brasilense	393	M D K N L Y D L P A E E	11/12	Bozouklian & Elmerich, 1986
C. acetobutylicum	367	V E A N I F A M T E Q E	2/12	Janssen et al., 1988
S. coelicolor	391	I D K D L Y E L A P E E	8/12	Rawlings - pers. comm.

The amino acids are in single letter code, and the positions of the amino acids in the relevant GS enzymes are indicated. Numbering of the residues begins with the start methionine at the N-terminal end. Those residues which are conserved, are boxed.

catalytic activity is observed upon oxidative modification of this histidine residue. This loss of catalytic activity is thought to be due to alteration of binding of divalent cations essential for activity and based on this and X-ray crystallographic studies, it has been proposed that this peptide constitutes part of the active site of the GS enzyme (Farber and Lavine, 1986; Almassy *et al.*, 1986).

A comparison of the residues around 270 to 274 from *E. coli* GS with other published nucleotide sequence-derived GS amino acid sequences and the *M. capsulatus* (Bath) sequence are presented in Figure 5:7. The GS enzymes from *S. typhimurium*, *T. ferrooxidans*, *Anabaena* 7120, *A. brasilense*, *C. acetobutylicum*, *S. coelicolor* as well as *M. capsulatus* (Bath) all possessed a histidine residue in a similar position to that in the *E. coli* GS monomer. The residues around this histidine in all the published prokaryotic GS enzymes are highly conserved with the *M. capsulatus* (Bath) GS enzyme displaying a 4 out of 6 residue match to the *E. coli* GS enzyme in this region, and a 6 out of 6 residue match to the *T. ferrooxidans* and *S. coelicolor* GS enzymes.

The hydropathy profile of the deduced amino acid sequence of the *M. capsulatus* (Bath) GS enzyme has also been determined (Figure 5:8). The profile of this plot did not show any long stretches of hydrophobic or hydrophilic amino acids, which was expected for a globular-soluble proteins.

ii) Prokaryotic and eukaryotic.

A comparison of eukaryotic and prokaryotic GS enzymes has previously been carried out by Rawlings *et al.*, (1987) and Janssen *et al.*, (1988). Rawlings and his co-workers showed that although the amino acid homology between the GS enzymes was only approximately 15%, the major part of this homology was located in five regions.

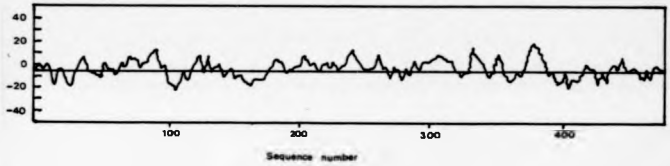
Figure 5:7 Comparison of amino acid sequences around the peptide, MHCHM, constituting part of the GS active site from published prokaryotic sequences.

Organism	Residue	Amino acid sequence	Match	Ref.
E. coli	270	M H C H M S	6/6	Miranda-Rios et al., 1987
S. typhimurium	269	M H C H M S	6/6	Janson et al., 1986
T. ferrooxidans	258	M H V H Q S	4/6	Rawlings et al., 1987
M. capsulatus (Bath)	269	M H V H Q S	4/6	This Work
Anabaena 7120	272	M H C H Q S	5/6	Tumer et al., 1983
A. brasilense	269	M H M H Q S	4/6	Bozouklian & Elmerich, 1986
C. acetobutylicum	243	M H V N M S	4/6	Janssen et al., 1988
S. coelicolor	265	M H V H Q S	4/6	Rawlings - pers. comm.

Amino acids are identified by the single-letter code, and the positions of the amino acid residues in the GS enzymes are indicated. Numbering of residues begins with the start methionine at the N-terminal end. Identical residues are boxed.

Figure 5:8 Hydropathic profile of the deduced polypeptide encoded by the *M. capsulatus* (Bath) *glnA* structural gene.

The hydropathic profile shown is based upon the scale of Kyte and Doolittle, (1982). A span of eleven consecutive residues was used.



Using the published prokaryotic/eukaryotic GS amino acid sequence comparison as a basis (Rawlings *et al.*, 1987; Janssen *et al.*, 1988), the amino acid sequences of the following GS enzymes were compared: *Phaseolis vulgaris*, Chinese hamster, Alfalfa, *Bradyrhizobium japonicum*, *E. coli*, *Anabaena* 7120, *T. ferrooxidans*, *S. typhimurium*, *C. acetobutylicum*, *A. brasilense*, *S. coelicolor* and *M. capsulatus* (Bath), (see Figure 5:9).

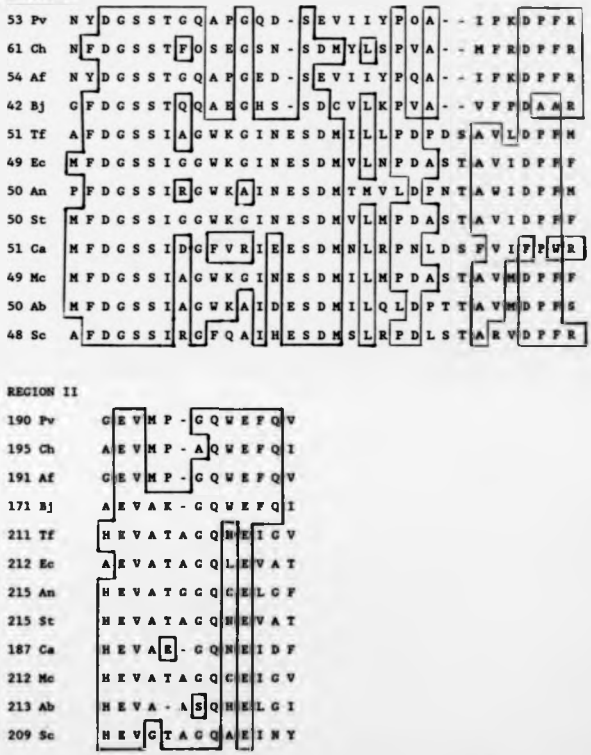
The data detailed in Figure 5:9 show that the five regions of homology in the GS polypeptide are highly conserved in both prokaryotic and eukaryotic GS enzymes from a number of organisms and that the GS enzyme of *M. capsulatus* (Bath) also exhibits this conservation of sequence.

5:3 Discussion

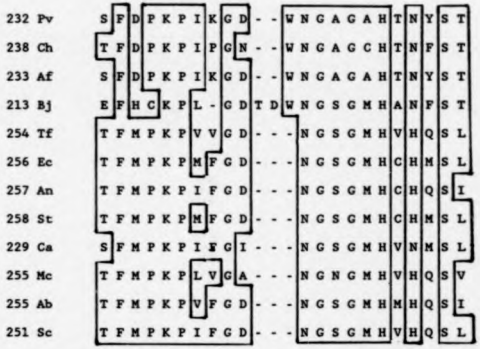
The nucleotide sequence of the *M. capsulatus* (Bath) *glnA* structural gene and flanking sequences have been determined. The *M. capsulatus* (Bath) GS monomer is encoded by a 1407 bp ORF. The M_r of the predicted polypeptide encoded by this region was 51,717, consisting of 468 amino acids. This is lower than the apparent M_r of 60,000 estimated by gel electrophoresis of the *M. capsulatus* (Bath) GS polypeptide, reported by Murrell and Dalton (1983(c)) and also of that observed on polyacrylamide gels from *in vitro* transcription/translation experiments with the plasmids pDC1, pDC2 and pDC100. (This work - see Chapter 4). A similar finding was reported for the *C. acetobutylicum* GS monomer in which the cloned plasmid-encoded GS polypeptide had an apparent M_r of 59,000, estimated by gel electrophoresis, whereas the nucleotide sequence derived GS polypeptide had an apparent M_r of 49,630 (Janssen *et al.*, 1988).

Murrell and Dalton proposed a decameric structure for the native *M. capsulatus* (Bath) GS enzyme, based on the apparent M_r's of both the native enzyme (M_r 617,000) and the GS monomer (M_r 60,000) (Murrell and Dalton, 1983(c)). The M_r of the nucleotide sequence derived GS polypeptide

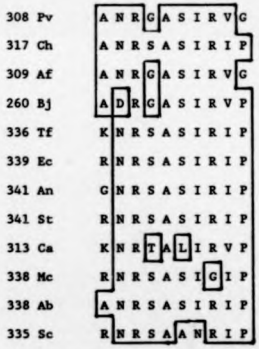
Figure 5:9 Comparison of amino acid sequences of five regions of homology of G8 enzymes from both prokaryotic and eukaryotic sources.



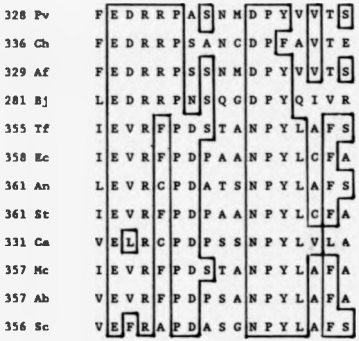
REGION III



REGION IV



REGION V



The amino acids are identified by the single letter code, and the positions of the amino acids in the GS enzyme indicated. Numbering of residues begins with the start methionine at the N-terminal end. Identical residues are boxed.

Key

Pv = *Phaseolis vulgaris*	An = *Anabaena* 7120
Ch = Chinese hamster	St = *Salmonella typhimurium*
Af = Alfalfa	Ca = *Clostridium acetobutylicum*
Bj = *Bradyrhizobium japonicum*	Mc = *Methylococcus capsulatus* (Bath)
Tf = *Thiobacillus ferrooxidans*	Ab = *Azospirillum brasilense*
Ec = *Escherichia coli*	Sc = *Streptomyces coelicolor*

presented here suggests a dodecameric structure for the *M. capsulatus* (Bath) GS enzyme as found for all other reported prokaryotic GS enzymes, with the exception of the *Clostridium pasteurianum* GS enzyme, which is reported to have a 20 subunit structure (Krishnan *et al.*, 1986).

The hydropathy profile of the *M. capsulatus* (Bath) *glnA* gene product (Figure 5:9) exhibits a pattern expected for globular-soluble protein with no long stretches of hydrophobic or hydrophilic residues.

The codon usage pattern of the *M. capsulatus* (Bath) *glnA* gene reflects the high reported G + C content of this organism (Whittenbury and Krieg, 1984), in which codon usage is strongly biased towards the use of codons in which G and C predominate. A comparison of the patterns of codon usage of *glnA* genes from *E. coli*, *S. typhimurium*, *T. ferrooxidans*, *Anabaena* 7120 and *M. capsulatus* (Bath) (Table 5:1) revealed a similar trend in codon usage for all the organisms with the exception of *Anabaena* 7120. The importance of studying patterns of codon usage is realised when determining the source of foreign DNA to express in a particular host, or when determining a host for expression of particular DNA. Due to the degeneracy in the genetic code, preferred codon usage may vary from organism to organism, and the amounts of particular cytoplasmic tRNA's is reflected by the frequency of occurrence of the respective codons in the genome (Ikemura, 1982). Therefore, the efficient expression of foreign DNA in any surrogate host would require a similar preferred codon usage. From these studies on preferred codon usage it would appear that, DNA from either *E. coli*, *S. typhimurium* or *T. ferrooxidans* would be efficiently translated in *M. capsulatus* (Bath), and that *M. capsulatus* (Bath) DNA would conversely be efficiently translated in these organisms. To gain a more accurate assessment of preferred codon usage in *M. capsulatus* (Bath), other genes from this organism will need to be examined.

The trend in codon usage patterns of *N. capsulatus* (Bath) *E. coli*, *S. typhimurium*, *T. ferrooxidans* and *Anabaena* 7120 *glnA* genes was paralleled by the level of nucleotide sequence homology between the *N. capsulatus* (Bath) *glnA* gene and the *glnA* genes from these organisms. Only a moderate degree of homology (46%) in the nucleotide sequence of the *glnA* genes from *N. capsulatus* (Bath) and *C. acetobutylicum* was observed, probably due to the low G + C content (28%) of *C. acetobutylicum*.

Initial hybridization analysis using the cloned *Anabaena* 7120 *glnA* gene as a gene probe revealed no detectable homology with any *N. capsulatus* (Bath) sequences even at very low stringencies (<20% homology) (see Chapter 3). However, subsequent nucleotide sequence comparison studies has revealed a moderate degree of homology (59.1%) between the two *glnA* sequences. Barros and his co-workers also reported a lack of detectable DNA homology from hybridization analysis (stringency <20%) between the *T. ferrooxidans* and *E. coli glnA* genes (Barros *et al.*, 1985). Comparative nucleotide sequence analysis of *E. coli* and *T. ferrooxidans glnA* genes revealed a 65.6% homology between the two respective genes. Similarly, Usadin and her colleagues reported a lack of detectable homology from hybridization analysis (stringency <20%) between *E. coli* and *C. acetobutylicum glnA* genes (Usdin *et al.*, 1986). However, a subsequent report by Janssen and colleagues revealed 49% homology at the nucleotide level between the respective *glnA* genes. A degree of caution is therefore required in interpretation of results obtained from hybridization analyses, and that 'no detectable homology' does not necessarily mean 'does not exist'!

The nucleotide sequence derived GS amino acid sequence comparison between *N. capsulatus* (Bath) GS and various prokaryotic GS sequences (Table 5:3) revealed a high degree of conservation of the GS polypeptide. This high degree of conservation was particularly notable in two regions of the GS monomer; namely the adenylylation and cation binding sites (around

residues 399 and 270 respectively in *E. coli*) (see Figures 5:6 and 5:7).

The tyrosine residue at position 399 in the *E. coli* GS monomer has previously been identified as the site of adenylylation (Heinrikson and Kingdon, 1971). This tyrosine residue is present in a similar position in the GS enzymes of *S. typhimurium*, *T. ferrooxidans*, *Anabaena* 7120, *A. brasilense* and *M. capsulatus* (Bath). The amino acid residues around this tyrosine residue exhibited a strong degree of homology in the GS enzymes of *E. coli*, *S. typhimurium*, *T. ferrooxidans*, *M. capsulatus* (Bath), *A. brasilense*, and to a lesser extent *Anabaena* 7120, *S. coelicolor*, and least of all, *C. acetobutylicum* (only 2 out of 12 residue match). Although *Anabaena* 7120 GS has a tyrosine residue in the correct position, it has an 8 out of 12 residue homology to the same region in the *E. coli* GS. The *Anabaena* GS is not adenylylated when cloned in *E. coli* (Fisher *et al.*, 1981), which could be a consequence of a different amino acid sequence surrounding the conserved tyrosine residue. The cloned *C. acetobutylicum* GS is also not adenylylated in *E. coli* (Usdin *et al.*, 1986) probably due to the presence of a phenylalanine residue in the place of the modifiable tyrosine and also a lack of homology with the *E. coli* GS in this region (only 2 out of 12 residues match). As the *M. capsulatus* (Bath) GS exhibits strong conservation with the same region of the *E. coli* GS around tyrosine 398 (11 out of 12 residues match), it is proposed that the cloned *M. capsulatus* (Bath) GS is adenylylated in *E. coli* as it is *in vivo* in *M. capsulatus* (Bath) (Murrell and Dalton, 1983(c)).

The second major region of conservation among the prokaryotic GS enzymes studied, was around the pentapeptide MHCHM (located from residues 270-274 in *E. coli* GS), which contains the oxidizable histidine residue involved in cation binding. This peptide is thought to constitute part of the active site of the enzyme (Farber and Levine, 1986; Almassy *et al.*, 1986). All the prokaryotic GS enzymes studied (as well as the eukaryotic GS enzymes - Figure 5:9 region III) have this histidine residue conserved,

together with moderately conserved surrounding residues.

The amino acid sequence of the *N. capsulatus* (Bath) GS was compared to a recently published analysis of eukaryotic and prokaryotic GS enzymes (Janssen *et al.*, 1988). In addition, the nucleotide sequence derived amino acid sequences of the GS enzyme from *A. brasilense* and *S. coelicolor* were also compared.

Rawlings and his co-workers had previously shown that the GS enzymes from both prokaryotes and eukaryotes had five highly conserved amino acid regions within their primary structure (Rawlings *et al.*, 1987). X-ray crystallographic studies on the unadenylylated GS from *S. typhimurium* showed that the active site was formed by two polypeptide chains (Almassy *et al.*, 1986). Subsequent studies revealed that, the five conserved amino acid regions within the GS enzymes of both prokaryotic and eukaryotic organisms, are all associated with the proposed GS active site. Regions II to V are β-strands closely associated with two Mn^{2+} cations of one subunit, whilst region I contains the tryptophan residue, which is thought to complete the active site formed between adjacent subunits (Rawlings *et al.*, 1987). These five regions were also highly conserved in the *N. capsulatus* (Bath) GS enzyme (see Figure 5:9).

Analysis of the regions surrounding the *N. capsulatus* (Bath) *glnA* coding region, has revealed the existance of three putative promoter sequences upstream of the *glnA* gene (P_1, P_2 and P_3 - Figure 5:3). The putative P_1 and P_2 promoter elements are similar to the classic -10 and -35 RNA polymerase-binding consensus sequences. The -10 region (Pribnow box) of the P_1 and P_2 promoters each had a 5 out of 6 nucleotide match to the consensus promoter, however, the -35 regions only displayed a 2 out of 6 and a 3 out of 6 match to the consensus respectively. A third putative promoter, P_3, was found located between P_1 and P_2. This promoter resembles the NtrA-dependent promoter consensus GG-N_{10}-GC motif between -24 and -12 (Dixon, 1984). In addition, a clearly identifiable NtrC binding site with

a 11 out of 15 nucleotide match to the consensus NtrC binding sequence (Dixon, 1984) was located 12 bp upstream of P_3 in the *N. capsulatus* (Bath) *glnA* leader region.

In the enteric organisms, *E. coli*, *K. pneumoniae* and *S. typhimurium*, the expression of *glnA* has been shown to be regulated by a pair of tandem promoters (Dixon, 1984; Reitzer and Magasanik, 1985; Kustu *et al.*, 1986). In *E. coli*, the *glnA*p1 promoter (promoter with the -35, -10 RNA polymerase-binding consensus sequence) lies approximately 100 bp upstream of the NtrA-dependant promoter, *glnA*p2 (Reitzer and Magasanik, 1985). In *N. capsulatus* (Bath) the *glnA* gene leader region appears to be regulated by tandem promoters, P_1 and P_3 similar to *glnA*p1 and P_2 similar to *glnA*p2. However, the relative positions of these promoter elements differ from the promoters in the *glnA* gene leader regions of the enteric bacteria. The position of the putative NtrC binding site within the *N. capsulatus* (Bath) *glnA* gene leader region also differs from that found in the enteric bacteria. In *E. coli*, *K. pneumoniae* and *S. typhimurium*, the Pribnow box of the *glnA*p1 promoter is flanked by a NtrC binding site, which is responsible for the repression of *glnA*p1 during growth in nitrogen-limiting conditions (Dixon, 1984; Reitzer and Magasanik, 1985; Kustu *et al.*, 1986). The *glnA* gene leader region of *T. ferrooxidans* has a *glnA*p1-type promoter but no *glnA*p2 type promoter. However, the Pribnow box of this *glnA*p1-type promoter is also flanked by an NtrC binding site as found in the enteric organisms studied (Rawlings *et al.*, 1987). The putative NtrC binding site within the *N. capsulatus* (Bath) *glnA* gene leader region flanks the Pribnow box of the *glnA*p1-type promoter P_1, which may act to repress expression from this promoter during growth in nitrogen-limiting conditions. In enteric organisms two high affinity NtrC binding sites lie >100 bp upstream of *glnA*p2 such that, during nitrogen-limiting conditions, NtrC-P binds to these sites and activates transcription from *glnA*p2. This activation is thought to occur by a looping of the intervening DNA between the binding

sites and *glnAp2* such that NtrC-P and σ^{54} RNA polymerase are in close proximity (reviewed in Dixon, 1988). In *N. capsulatus* (Bath) the putative NtrC binding site is located within one turn of the helix to the *glnAp2* like promoter, P_2. In this position activation of P_2 by NtrC-P would not require a looping of the intervening DNA during nitrogen-limited growth. The activation of P_2 in *N. capsulatus* (Bath) would be different from any other system thus far studied and therefore requires further analysis i.e. high resolution S1 nuclease mapping of transcripts, in order to identify the nature of activation and repression of the *N. capsulatus* (Bath) *glnA* structural gene.

Expression analysis of the cloned *N. capsulatus* (Bath) *glnA* gene in *E. coli* Ntr^+ and Ntr^- strains, indicated a Ntr^+ phenotype was required for its regulation under different conditions of nitrogen availability (see Chapter 4). The data presented here suggests that the presence of the putative promoters P_1, P_2 and P_3, as well as a putative NtrC binding site in the *N. capsulatus* (Bath) *glnA* gene leader region effects regulation of *glnA* in *N. capsulatus* (Bath) via an analogous Ntr system to that found in the enteric bacteria. Evidence to support the presence of analogous *ntr* genes in the *N. capsulatus* (Bath) genome is outlined in Chapter 6 of this thesis.

Analysis of the 3'-flanking region of the *N. capsulatus* (Bath) *glnA* gene revealed the existence of a small ORF encoding a polypeptide of M_r 7022. This ORF lies some 100 bp downstream of the *glnA* stop codon and is possibly co-transcribed with *glnA*, as no recognizable promoter or *rho* independent terminator sequences are present in the intergenic region. The function of the polypeptide encoded by this ORF remains to be elucidated, as a homology search at both the nucleotide and amino acid levels using the Microgenie DNA and protein databanks, failed to reveal any homology with any sequences within these extensive databanks.

Contrastingly, in the Gram positive obligate anaerobe, *C. acetobutylicum*, there is no evidence as to the existence of a global regulatory *ntr* system

(Usdin *et al.*, 1986). The cloned *glnA* gene from this organism was expressed and regulated however, by levels of nitrogen from its own regulatory region in *E. coli*. Subsequent molecular analysis of the DNA fragment containing the *C. acetobutylicum glnA* gene by Janssen and co-workers, revealed the existence of an extensive stretch of inverted repeat sequences and a putative promoter, downstream of the *glnA* structural gene. Deletion of the putative promoter and downstream inverted repeat sequences affected the regulation of GS such that, levels were reduced approximately five fold under nitrogen-limiting conditions but repression of GS levels in cells grown under nitrogen-excess conditions were unaffected. The downstream putative promoter was orientated toward the 3' end of the *glnA* structural gene. This promoter was followed by a 43-base region which exhibited strong homology with a 43-base sequence situated at the start of the *glnA* gene. Transcription from this promoter would produce an antisense RNA, which could form an RNA-RNA hybrid structure with the 5' region of the *glnA* mRNA. This in turn would prevent translation of the *glnA* mRNA, as both the Shine-Dalgarno sequence and initiation codon are located within the base-paired region. Transcription of the region immediately downstream of the *C. acetobutylicum glnA* gene containing a 158 bp stretch of inverted repeat sequences, would produce a mRNA with the potential to form a number of stem-loop structures. It was postulated that the resulting stem loops may play a role in either mRNA termination or stabilization (Janssen *et al.*, 1988).

The results obtained from study of the *C. acetobutylicum glnA* gene region revealed that DNA sequences downstream of the *glnA* gene had a regulatory role. This *glnA* downstream region in *C. acetobutylicum* differed from that found in the enteric bacteria (Merrick, 1988(a)).

Analysis of the 3'-flanking region of the *K. capsulatus* (Bath) *glnA* gene did not reveal any sequences similar to that found in the enteric bacteria or *C. acetobutylicum*. However, the evidence presented here

indicates the presence of an analogous *ntr* system to that in the enteric bacteria, in *N. capsulatus* (Bath). However, unlike that in the enteric bacteria, the putative *ntrB* and *ntrC* genes of *N. capsulatus* (Bath) are unlinked to *glnA* (cf. *R. meliloti* - Szeto *et al.*, 1987). Further evidence in support of the existence and location of *ntr*-like genes in the *N. capsulatus* (Bath) genome is presented in Chapter 6 of this thesis.

CHAPTER 6

Nitrogen regulatory genes in *Methylococcus capsulatus* (Bath) and other obligate methanotrophs.

6:1 Introduction.

The expression and nucleotide sequence of the cloned *N. capsulatus* (Bath) *glnA* structural gene has revealed the probable existence in this organism of an analogous nitrogen regulatory (*ntr*) system to that found in enteric, as well as a number of non-enteric bacteria. These studies were therefore designed to investigate the presence of *ntr*-like genes in *N. capsulatus* (Bath) and to determine their location on the *N. capsulatus* (Bath) genome. The existence of *ntr*-like genes in Type I and Type II obligate methanotrophs was also investigated.

6:2 Results.

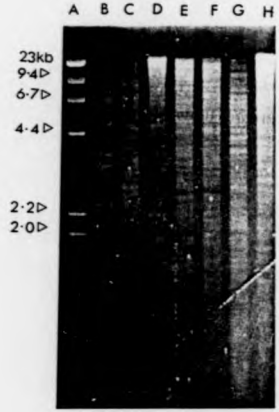
6:2:1 Heterologous hybridisation studies with the *K. pneumoniae ntrB* and *ntrC* genes as hybridisation probes.

N. capsulatus (Bath) genomic DNA was digested to completion with various restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at low stringency with ^{32}P-labelled *K. pneumoniae ntrB* (1 kb *Kpn*I insert of pSM10), or *ntrC* (1.5 kb *Eco*RI-*Hind*III fragment of pMD114 - see Figure 6:1). The resulting autoradiograph patterns are shown in Figures 6:2 and 6:3 respectively, and summarized on Table 6:1.

A number of distinct *N. capsulatus* (Bath) genomic fragments exhibited homology to both *K. pneumoniae ntrB* and *ntrC* gene probes. Specific *ntrB*

Figure 6:1 Heterologous DNA probes used in this study.

Klebsiella pneumoniae	*ntrB*	-	1 kb *Kpn*I fragment of pSM10
Klebsiella pneumoniae	*ntrC*	-	1.5 kb *Eco*RI - *Hin*dIII fragment of pND114
Azotobacter vinelandii	*glnA-ntrC*	-	6 kb *Eco*RI fragment of pAT523
Azotobacter vinelandii	*ntrA*	-	2 kb *Eco*RI-*Cla*I fragment of pAT705
Klebsiella pneumoniae	*ntrA*	-	1.9 kb *Cla*I fragment of pNM17
Escherichia coli	*rpoD*	-	1.5 kb *Hin*dIII fragment of pNM26
Rhizobium leguminosarum	*glnB*	-	0.4 kb *Eco*RI-*Bam*HI fragment of pAH3
Escherichia coli	*glnB*	-	1.6 kb *Eco*RI-*Sal*I fragment of pAH5


A B C D E F G H
23kb
9·4▷
6·7▷
4·4▷
2·2▷
2·0▷

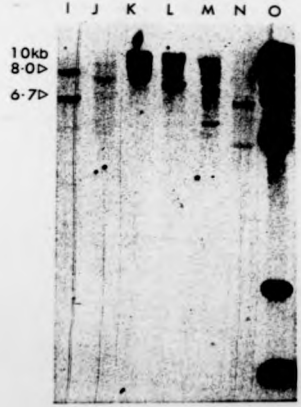

I J K L M N O
10kb
8·0▷
6·7▷

Figure 6:2 Identification of *M. capsulatus* (Bath) genomic sequences exhibiting homology to the *K. pneumoniae ntrB* gene probe.

Key to tracks:

A	λ DNA restricted with *Hin*dIII
B	*M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
C	*M. capsulatus* (Bath) genomic DNA restricted with *Sal*I
D	*M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII
E	*M. capsulatus* (Bath) genomic DNA restricted with *Kpn*I
F	*M. capsulatus* (Bath) genomic DNA restricted with *Xho*I
G	*M. capsulatus* (Bath) genomic DNA restricted with *Pst*I
H	*K. pneumoniae* genomic DNA restricted with *Kpn*I

Tracks 1 to 0 are autoradiographs of corresponding tracks B to H probed at low stringency with *K. pneumoniae ntrB* gene probe (pSM10 - 1 kb *Kpn*I fragment).

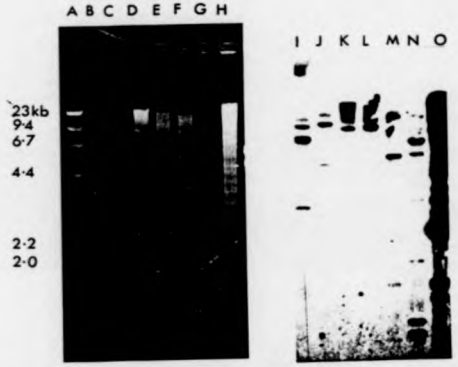

A B C D E F G H
I J K L M N O
23kb
9·4
6·7
4·4
2·2
2·0

Figure 6.3 Identification of *M. capsulatus* (Bath) genomic sequences exhibiting homology to the *K. pneumoniae ntrC* gene probe.

Key to tracks:

A λ DNA restricted with *Hind*III

B *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI

C *M. capsulatus* (Bath) genomic DNA restricted with *Sal*I

D *M. capsulatus* (Bath) genomic DNA restricted with *Hind*III

E *M. capsulatus* (Bath) genomic DNA restricted with *Kpn*I

F *M. capsulatus* (Bath) genomic DNA restricted with *Xho*I

G *M. capsulatus* (Bath) genomic DNA restricted with *Pst*I

H *K. pneumoniae* genomic DNA restricted with *Kpn*I

Tracks I to O are autoradiographs of corresponding tracks B to H probed at low stringency with *K. pneumoniae ntrC* gene probe (pMD114 1.5 kb *Eco*RI - *Hind*III fragment).

Table 6:1 Sizes of *K. capsulatus* (Bath) genomic DNA restriction fragments that exhibit homology to *K. pneumoniae ntrB* and *ntrC* gene probes.

This table summarizes the data presented in Figures 6:2 and 6:3.

Restriction Endonuclease	*EcoR*I		*Sal*I		*Hind*III	
Homology with	*ntrB*	*ntrC*	*ntrB*	*ntrC*	*ntrB*	*ntrC*
Fragment size (kb)	6.7	8.0	9.0	9.0	10.0	10.0
	3.0	6.7	7.8	5.0		
		3.0				

Restriction Endonuclease	*Kpn*I		*Xho*I		*Pst*I	
Homology with	*ntrB*	*ntrC*	*ntrB*	*ntrC*	*ntrB*	*ntrC*
Fragment size (kb)	10.2	10.2	5.0	5.0	6.2	6.2
	7.6	7.6		1.1		

hybridizing fragments corresponded in size to specific *ntrC* homologues, suggesting a possible linkage of these two genes in *H. capsulatus* (Bath).

Previous studies have shown that the products of the *K. pneumoniae ntrC* and *nifA* genes exhibit considerable homology at the amino acid level (Buikema *et al.*, 1985; Drummond *et al.*, 1986). Due to this homology, a Southern blot containing *H. capsulatus* (Bath) genomic DNA was probed with the *K. pneumoniae nifA* gene (kindly donated by C. Oakley) in order to distinguish true *ntrC* homologues from *nifA* homologues. No cross-hybridizing bands were detected (data not shown).

In order to confirm the results obtained using the *K. pneumoniae ntrC* gene as hybridization probe, a Southern blot containing *H. capsulatus* (Bath) genomic restriction digests was probed at low stringency with ^{32}P-labelled *A. vinelandii glnA-ntrC* gene region (6 kb *Eco*RI fragment of pAT523 - see Figure 6:1). The resulting autoradiograph patterns are shown in Figure 6:4 and summarized on Table 6:2. Specific *H. capsulatus* (Bath) restriction fragments which exhibited homology to this probe, corresponded in size to *K. pneumoniae ntrC* (Figure 6:3) and *glnA* (see Chapter 3, Figure 3:3) homologues.

6.2:2 Screening of a *H. capsulatus* (Bath) cosmid gene library with the *K. pneumoniae ntrC* gene.

Heterologous hybridization studies (described above) established the presence of an *ntrC*-like sequence in the *H. capsulatus* (Bath) genome. In order to isolate the putative *H. capsulatus* (Bath) *ntrC* structural gene, a *H. capsulatus* (Bath) cosmid gene library was constructed and subsequently screened with the *K. pneumoniae ntrC* gene as hybridization probe.

The construction of the *H. capsulatus* (Bath) pVK100 cosmid gene library is outlined in Section 2:15, Chapter 2. Screening of the cosmid library was carried out with ^{32}P-labelled *K. pneumoniae ntrC* at low

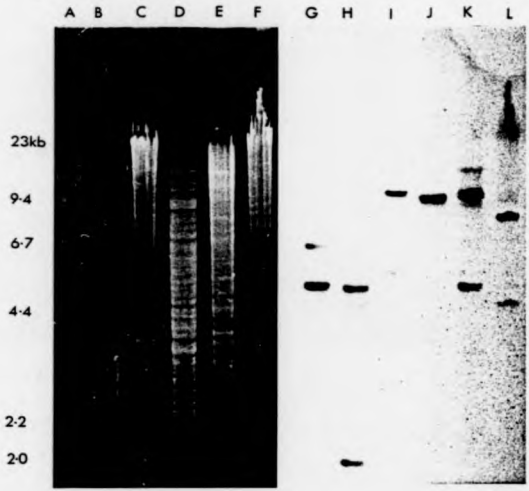

A B C D E F G H I J K L
23kb
9·4
6·7
4·4
2·2
2·0

Figure 6:4 Identification of *H. capsulatus* (Bath) genomic sequences exhibiting homology to the *A. vinelandii glnA-ntrC* DNA probe.

H. capsulatus (Bath) genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted and the resulting filter probed at a stringency allowing approximately 60% bp mismatch with the *Azotobacter vinelandii glnA-ntrC* gene probe (pAT523 6 kb *Eco*RI fragment).

Key to tracks:

A *H. capsulatus* (Bath) genomic DNA restricted with *Eco*RI

B *H. capsulatus* (Bath) genomic DNA restricted with *Sal*I

C *H. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII

D *H. capsulatus* (Bath) genomic DNA restricted with *Pst*I

E *H. capsulatus* (Bath) genomic DNA restricted with *Xho*I

F *H. capsulatus* (Bath) genomic DNA restricted with *Kpn*I

Tracks G to L are autoradiographs of corresponding tracks A to F probed with the *A. vinelandii* gene probe.

Table 6:2 Sizes of *M. capsulatus* (Bath) genomic DNA restriction fragments which exhibit homology to *A. vinelandii* *glnA-nt.C* gene region.

This table summarizes the data presented in Figure 6:4.

Restriction Endonuclease	*Eco*RI	*Sal*I	*Hin*dIII	*Pst*I	*Xho*I	*Kpn*I
Fragment size (kb)	6.7	5.0	10.0	10.0	10.0	9.5
	5.2	4.5	5.6	1.1	5.0	4.6
	3.0	2.2				

stringency. Three cosmid clones were isolated which exhibited homology to the *ntrC* gene probe, and subsequent restriction analysis of these cosmids revealed them to contain identical *N. capsulatus* (Bath) inserts (data not shown). One of these cosmids was chosen for further study and was designated pCOS1.

6:2:3 Restriction analysis of pCOS1.

Cosmid pCOS1 DNA was digested with a variety of restriction endonucleases and the resulting fragments fractionated by agarose gel electrophoresis. The resulting restriction pattern is shown in Figure 6:5 and summarized in Table 6:3.

A comparison between restriction patterns of pCOS1 and that of pDC1 (see Figure 3:8, Chapter 3) revealed fragments of corresponding size, common to both recombinants. The presence of the *N. capsulatus* (Bath) *glnA* structural gene within pCOS1 was confirmed by hybridization analysis. *N. capsulatus* (Bath) genomic DNA and pCOS1 DNA were digested to completion with a variety of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with ^{32}P-labelled *K. pneumoniae glnA* fragment. The resulting autoradiograph pattern is shown in Figure 6:6.

The results obtained clearly demonstrated that pCOS1 contained the *N. capsulatus* (Bath) *glnA* structural gene. Subsequent analysis of the *N. capsulatus* (Bath) restriction fragments present in pCOS1 and the *N. capsulatus* (Bath) genomic fragments exhibiting homology to the pDC1 insert (Figure 3:1, Chapter 3) revealed pCOS1 insert DNA to completely overlap the *N. capsulatus* (Bath) DNA present in pDC1/pDC2 (see Figure 6:7).

Figure 6.5 Restriction enzyme analysis of pCOS1.

Cosmid pCOS1 DNA was digested with the restriction endonucleases indicated and the resulting fragments fractionated by agarose gel electrophoresis.

Key to tracks:

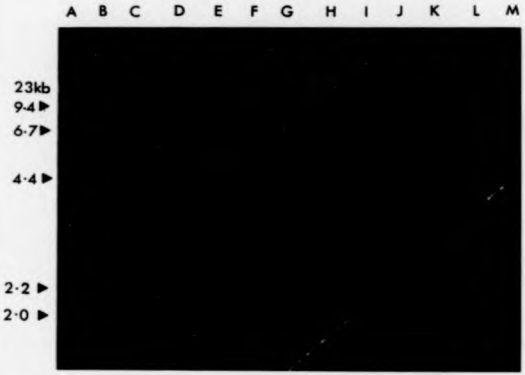
A	pCOS1 DNA restricted with *Kpn*I
B	pCOS1 DNA restricted with *Eco*RI
C	pCOS1 DNA restricted with *Hin*dIII
D	λ DNA restricted with *Hin*dIII

A B C D

Table 6:3 Restriction endonuclease analysis of pCOS1.

This table summarizes the data presented in Figure 6:5.

Restriction Endonuclease	*Kpn*I	*Eco*RI	*Hind*III
Fragment size (kb)	>23.0	22.0	>23.0
	4.8	9.0	10.0
	3.4	5.2	5.6
		3.0	


A B C D E F G H I J K L M
23kb
9·4
6·7
4·4
2·2
2·0

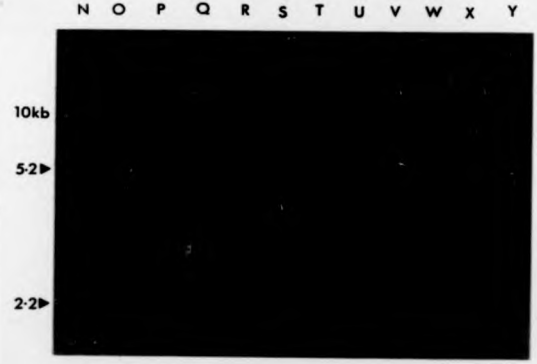

N O P Q R S T U V W X Y
10kb
5·2
2·2

Figure 6:6 **Identification of *glnA* within pCOS1 by heterologous hybridization with the *K. pneumoniae glnA* gene probe.**

M. capsulatus (Bath), *K. pneumoniae* genomic and pCOS1 DNA were restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted and the resulting filter probed at a stringency allowing approximately 40% bp mismatch with the *K. pneumoniae glnA* gene probe (pAM51 0.9 kb *Eco*RI fragment).

Key to tracks:

A	λ DNA restricted with *Hin*dIII
B	*M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
C	*M. capsulatus* (Bath) genomic DNA restricted with *Sal*I
D	*M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII
E	*M. capsulatus* (Bath) genomic DNA restricted with *Kpn*I
F	*M. capsulatus* (Bath) genomic DNA restricted with *Xho*I
G	*M. capsulatus* (Bath) genomic DNA restricted with *Pst*I
H	*K. pneumoniae* genomic DNA restricted with *Kpn*I
I	pDC1 DNA restricted with *Eco*RI
J	pCOS1 DNA restricted with *Kpn*I
K	pCOS1 DNA restricted with *Eco*RI
L	pCOS1 DNA restricted with *Hin*dIII
M	pCOS1 DNA restricted with *Xho*I

Tracks N to Y are autoradiographs of corresponding tracks B to M probed with the *K. pneumoniae* gene probe.

pCOS 1

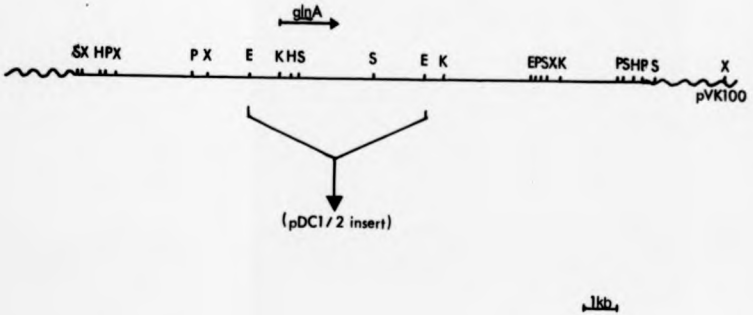


Figure 6:7 Restriction endonuclease map of pCOS1.

Key:

E	*Eco*RI
S	*Sal*I
X	*Xho*I
P	*Pst*I
H	*Hind*III
K	*Kpn*I

6:2:4 Determination of *glnA-ntrC* linkage in *N. capsulatus* (Bath).

In the Enterobacteriaceae, *glnA* and *ntrC* are linked in the form of a complex operon (McFarland *et al.*, 1981; Espin *et al.*, 1982; Fahel *et al.*, 1982; Rothman *et al.*, 1982). Linkage of *glnA* and *ntrC* has also been determined in the non-enteric organisms, *T. ferrooxidans*, *A. vinelandii* and *B. pertussis* (Barros *et al.*, 1985; Toukdarian and Kennady, 1986; Brownlie *et al.*, 1986).

Previous studies (Section 6:2:3) have determined the presence of *glnA* on the *ntrC* homologue, pCOS1. In order to determine the relative position of *glnA* and *ntrC* within the *N. capsulatus* (Bath) genome, pCOS1 DNA was digested to completion with a variety of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted and probed with ^{32}P-labelled *A. vinelandii glnA-ntrC* region (6 kb *Eco*RI fragment of pAT523 - see Figure 6:1). The resulting autoradiograph pattern is shown in Figure 6:8 and summarized on Table 6:4.

Certain hybridizing fragments corresponded to the *glnA* homologues detailed in Figure 6:6, whilst other hybridizing fragments corresponded to the *ntrC* homologues detailed in Figure 6:3. The 10 kb *Hin*dIII *N. capsulatus* (Bath) DNA fragment from pCOS1, which exhibited homology to both *glnA* and *ntrC* gene probes was chosen for further analysis. The 10 kb *Hin*dIII fragment overlaps pDC1, such that it contains all but the 5' portion of the *N. capsulatus* (Bath) *glnA* gene (see Figure 6:7).

Cosmid pCOS1 DNA was digested to completion with the restriction endonuclease *Hin*dIII, fractionated by agarose gel electrophoresis and the 10 kb *Hin*dIII fragment purified by extraction from the gel matrix by electroelution. The purified fragment was labelled with ^{32}P-dCTP by nick translation and used to probe a Southern blot containing various restriction digests of *N. capsulatus* (Bath) genomic DNA at high stringency. The resulting autoradiograph pattern is shown in Figure 6:9 and summarized

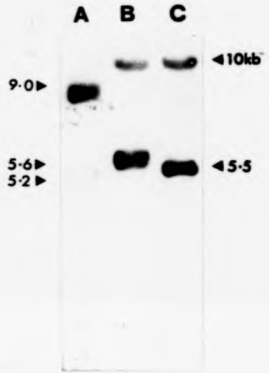

A
B
C
10kb
9·0
5·6
5·2
5·5

Figure 6:8 Identification of *glnA* and *ntrC* homologues within pCOS1 using the *A. vinelandii glnA-ntrC* region as heterologous hybridisation probe.

Cosmid pCOS1 DNA was restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted and probed with the *glnA-ntrC* region from *A. vinelandii* (6 kb *Eco*RI fragment from pAT523).

Key to tracks:

A Autoradiograph of pCOS1 restricted with *Eco*RI and probed with *A. vinelandii glnA-ntrC*.

B Autoradiograph of pCOS1 restricted with *Hin*dIII and probed with *A. vinelandii glnA-ntrC*.

C Autoradiograph of pCOS1 restricted with *Xho*I and probed with *A. vinelandii glnA-ntrC*.

Table 6:4 Sizes of pCO81 restriction fragments which exhibit homology to *A. vinelandii glnA-ntrC* gene probe.

This table summarizes the data presented in Figure 6:8.

Restriction Endonuclease	*Eco*RI	*Hin*dIII	*Xho*I
Fragment size (kb)	9.0	10.0	10.0
	5.2	5.6	5.5

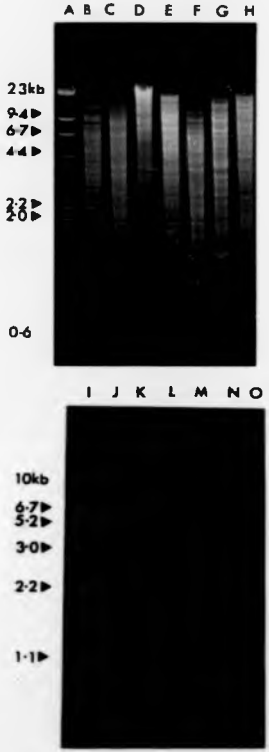

A B C D E F G H
23kb
9·4
6·7
4·4
2·3
2·0
0·6
I J K L M N O
10kb
6·7
5·2
3·0
2·2
1·1

Figure 4.9 Probing of *M. capsulatus* (Bath) genomic digests with the pCOS1 10 kb *Hin*dIII fragment.

M. capsulatus (Bath) genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted and probed at high stringency (assuming an approximate < 10% bp mismatch) with pCOS1 10 kb *Hin*dIII fragment.

Key to tracks:

A λ DNA restricted with *Hin*dIII

B *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI

C *M. capsulatus* (Bath) genomic DNA restricted with *Sal*I

D *M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII

E *M. capsulatus* (Bath) genomic DNA restricted with *Xpn*I

F *M. capsulatus* (Bath) genomic DNA restricted with *Pst*I

G *M. capsulatus* (Bath) genomic DNA restricted with *Cla*I

H *M. capsulatus* (Bath) genomic DNA restricted with *Xho*I

Tracks I to O are autoradiographs of corresponding tracks B to H probed with pCOS1 10 kb *Hin*dIII fragment.

Table 6:5 Sizes of *M. capsulatus* (Bath) genomic DNA restriction fragments that exhibit homology to the pCOS1 10 Kb *Hin*dIII fragment probe.

This table summarizes the data presented in Figure 6:9.

Restriction Endonuclease	*Eco*RI	*Sal*I	*Hin*dIII	*Kpn*I	*Pst*I	*Cla*I	*Xho*I
Fragment size (kb)	6.7	7.2	10.0	10.0	10.0	6.0	10.0
	5.2	5.0		4.6	6.2	2.45	5.0
	3.0	2.5		3.0	2.45	0.9	
		2.2			1.1	0.5	

on Table 6:5. Specific *N. capsulatus* (Bath) genomic DNA fragments which exhibited homology to this probe, corresponded in size to the *glnA* and *ntrC* homologues determined previously.

In order to further aid the mapping process, and in turn determine the location of the *N. capsulatus* (Bath) *ntrC* gene within pCOS1, the 5.6 kb *Hin*dIII fragment which contains the 5' end of the *N. capsulatus glnA* gene (see Figure 6:7), and the 5.5 kb *Xho*I fragment of pCOS1 (which exhibits homology to *A. vinelandii ntrC* (see Figure 6:8)), were used as homologous hybridization probes to Southern blots containing various restriction digests of *N. capsulatus* (Bath) genomic DNA and pCOS1. The resulting autoradiograph patterns are shown in Figures 6:10 and 6:11 respectively, and summarized on Table 6:6.

The data obtained from Southern hybridization and restriction endonuclease analysis enabled the determination of the pCOS1 restriction map (Figure 6:12). The localization of the *N. capsulatus* (Bath) *ntrC*-like gene within pCOS1 was achieved by comparative Southern hybridization analysis. For example, the *N. capsulatus* (Bath) 10 kb *Hin*dIII fragment of pCOS1, when used as a homologous hybridization probe against *N. capsulatus* (Bath) genomic digests, exhibited homology to a number of specific restriction fragments which corresponded in size to previously determined *ntrC* homologues (see Figure 6:3, 6:8 and 6:9). Specific restriction fragments exhibiting homology to the pCOS1 5.5 kb *Xho*I fragment also corresponded in size with the predetermined *ntrC* homologues (see Figure 6:11). However, the 5.5 kb pCOS1 *Xho*I fragment, when used as a hybridization probe exhibited homology to a single 5 kb *N. capsulatus* (Bath) *Xho*I genomic fragment, suggesting the presence of vector (pVK100) sequences and therefore occupying a terminal position within pCOS1. Subsequent analysis of pCOS1 fragments exhibiting homology to this probe showed this to be the case. The 5.5 kb pCOS1 *Xho*I fragment also exhibited homology to the 10 kb *Hin*dIII fragment within pCOS1 and also in the


A B C D E F G H I J K L M
23kb
9·4
6·7
4·4
2·2
2·0
0·6
N O P Q R S T U V W X Y
6·8
5·2
3·0
1·1

Figure 6:10 Mapping of pCOS1 by homologous hybridisation analysis using the pCOS1 5.6 kb *Hin*dIII fragment to probe *M. capsulatus* (Bath) genomic and pCOS1 restriction digests.

M. capsulatus (Bath) genomic and pCOS1 DNA were restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at high stringency (assuming an approximate < 10% bp mismatch).

Key to tracks:

A λ DNA restricted with *Hin*dIII

B *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI

C *M. capsulatus* (Bath) genomic DNA restricted with *Sal*I

D *M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII

E *M. capsulatus* (Bath) genomic DNA restricted with *Pst*I

F *M. capsulatus* (Bath) genomic DNA restricted with *Kpn*I

G *M. capsulatus* (Bath) genomic DNA restricted with *Xho*I

H pDC1 DNA restricted with *Sal*I

I pCOS1 DNA restricted with *Eco*RI

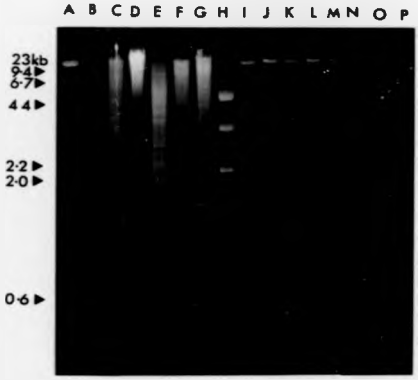
J pCOS1 DNA restricted with *Hin*dIII

K pCOS1 DNA restricted with *Sal*I

L pCOS1 DNA restricted with *Xho*I

M pCOS1 DNA restricted with *Pst*I

Tracks M to Y are autoradiographs of corresponding tracks B to M probed with pCOS1 5.6 kb *Hin*dIII fragment.


A B C D E F G H I J K L M N O P
23kb
9·4
6·7
4·4
2·2
2·0
0·6


Q R S T U V W X Y Z a b c d e
10kb
5·0
4·5
2·5
1·1
1·5

Figure 6:11 Mapping of pCOS1 by homologous hybridization analysis using the pCOS1 5.5 kb *Xho*I fragment to probe *M. capsulatus* (Bath) genomic and pCOS1 restriction digests.

M. capsulatus (Bath) genomic and pCOS1 DNA were restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at high stringency (assuming an approximate < 10% bp mismatch).

Key to tracks:

A	λ DNA restricted with *Hin*dIII
B	*M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
C	*M. capsulatus* (Bath) genomic DNA restricted with *Sal*I
D	*M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII
E	*M. capsulatus* (Bath) genomic DNA restricted with *Pst*I
F	*M. capsulatus* (Bath) genomic DNA restricted with *Kpn*I
G	*M. capsulatus* (Bath) genomic DNA restricted with *Xho*I
H	pDC1 DNA restricted with *Sal*I
I	pCOS1 DNA restricted with *Eco*RI
J	pCOS1 DNA restricted with *Hin*dIII
K	pCOS1 DNA restricted with *Sal*I
L	pCOS1 DNA restricted with *Xho*I
M	pCOS1 DNA restricted with *Pst*I
N	pCOS1 DNA restricted with *Kpn*I
O	pBR325 DNA
P	pMD114 DNA restricted with *Eco*RI and *Hin*dIII

Tracks Q to e are autoradiographs of corresponding tracks B to P probed with pCOS1 5.5 kb *Xho*I fragment. No discernible fragments are observed in tracks Q and c due to lack of transfer of DNA to nitrocellulose and nuclease activity respectively.

Table 6:6 Sizes of *M. capsulatus* (Bath) genomic and pCOS1 DNA which exhibit homology to the pCOS1 5.6 kb *Hin*dIII and 5.5 kb *Xho*I fragment probes.

This table summarizes the data presented in Figures 6:10 and 6:11.

Homology with pCOS1 5.6 kb *Hin*dIII fragment probe.

	M. capsulatus (Bath) genomic					
Restriction Endonuclease	*Eco*RI	*Sal*I	*Hin*dIII	*Pst*I	*Kpn*I	*Xho*I
Fragment size (kb)	6.8	7.5	5.6	10.0	10.5	10.0
	5.2	4.5		2.6	4.6	2.7
	3.0					1.1
						0.56

	pCOS1				
Restriction Endonuclease	*Eco*RI	*Hin*dIII	*Sal*I	*Xho*I	*Pst*I
Fragment size (kb)	5.2	5.6	4.5	10.0	10.0
	3.0			2.7	2.6
	2.6			1.1	
				0.56	

Homology with pCOS1 5.5 kb *Xho*I fragment probe.

M. capsulatus (Bath) genomic

Restriction Endonuclease	*Eco*RI	*Sal*I	*Hin*dIII	*Pst*I	*Kpn*I	*Xho*I
Fragment size (kb)	DNA not	5.0	10.0	2.4	10.0	5.0
	transferred to	2.4		1.1		
	nitrocellulose	1.4				

pCOS1

Restriction Endonuclease	*Eco*RI	*Hin*dIII	*Sal*I	*Xho*I	*Pst*I
Fragment size (kb)	9.0	>23.0	23.0	5.5	>23.0
		10.0	2.4		2.4
		2.65	1.6		1.1

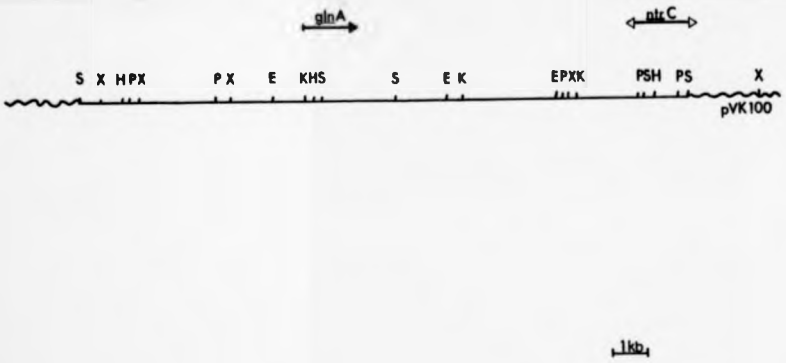

glnA
ntrC
S X HPX
P X
E
KHS
S
E K
EPXK
PSH PS
X
pVK100
1kb

Figure 6:12 Location of the *M. capsulatus* (Bath) *glnA* and putative *ntrC* genes within pCOS1.

Key:

E	*Eco*RI
S	*Sal*I
X	*Xho*I
P	*Pst*I
H	*Hin*dIII
K	*Kpn*I

 *ntrC* hybridizing region.

M. capsulatus (Bath) genome, together with the *K. pneumoniae ntrC* gene carried on the *EcoRI-HindIII* insert of pMD114 (see Figure 6:11). This data suggests that the *M. capsulatus* (Bath) *ntrC*-like gene is unlinked to *glnA* and lies some 8.5 kb downstream of *glnA* in this organism i.e. occupies a terminal position within the *M. capsulatus* (Bath) insert of pCOS1 (see Figure 6:12).

6:2:5 Complementation analysis of pCOS1.

The ability of pCOS1 to complement *E. coli* and *K. pneumoniae* (*glnA*, *ntrB*, *ntrC*) deletion mutants was determined.

Competent cells of *E. coli* ET8894 and *K. pneumoniae* UNF1848 were transformed with either the cosmids pCOS1 or pVK100 (control). Transformants were initially selected on LB agar containing kanamycin and glutamine (500 $\mu g\ ml^{-1}$). The ET8894 transformants were subsequently replica plated onto M9 minimal glucose agar containing kanamycin and either 0.1% (w/v) NH_4Cl (for $GlnA^+$ selection) or 0.2% (w/v) arginine (for Ntr^+ selection) as sole nitrogen source. UNF1848 transformants were subsequently replica plated onto NFDM agar containing kanamycin, histidine (25 $\mu g\ ml^{-1}$), and 0.1% (w/v) NH_4Cl or 0.2% (w/v) arginine as sole nitrogen source for growth. The ability of UNF1848 transformants, harbouring either pCOS1 or pVK100 to grow diazotrophically was tested on plates containing NFDM agar with kanamycin and histidine (25 $\mu g\ ml^{-1}$). *K. pneumoniae* transformants being tested for diazotrophic growth were incubated anaerobically under an atmosphere of nitrogen, at 30°C (see Table 6:7).

The cosmid pCOS1 enabled *E. coli* ET8894 to grow on 0.1% (w/v) NH_4Cl but not on 0.2% (w/v) arginine, as sole nitrogen source. *K. pneumoniae* UNF1848 cells harbouring pCOS1 were able to grow on 0.1% (w/v) NH_4Cl, but did not allow diazotrophic growth or growth on NFDM containing 0.2% (w/v) arginine as sole nitrogen source. The positive control used in all these

Table 6;7 Complementation of *E. coli* and *K. pneumoniae* (*glnA-ntrC*) deletion mutants with pCOS1 and its derivative, pDC110.

Organism	Nitrogen source for growth: 0.1% (w/v) NH_4Cl	0.2% (w/v) arginine	atmospheric N_2	Phenotype
E. coli				
ET8894	-	-	ND	$GlnA^-$ Ntr^-
ET8894/pCOS1	+	-	ND	$GlnA^+$ Ntr^-
ET8894/pDC110	+	-	ND	$GlnA^+$ Ntr^-
ET8894/pAT523	+	+	ND	$GlnA^+$ Ntr^+
ET8894/pVK100	-	-	ND	$GlnA^-$ Ntr^-
ET8556	+	-	ND	$GlnA^+$ Ntr^-
ET8556/pDC110	+	-	ND	$GlnA^+$ Ntr^-
ET8556/pAT523	+	+	ND	$GlnA^+$ Ntr^+
K. pneumoniae				
UNF1848	-	-	-	$GlnA^-$ Ntr^-
UNF1848/pCOS1	+	-	-	$GlnA^+$ Ntr^-
UNF1848/pVK100	-	-	-	$GlnA^-$ Ntr^-
UNF1848/pAT523	+	+	+	$GlnA^+$ Ntr^+

Key + = growth - = no growth ND = Not determined

experiments was pAT523 which contains the *glnA-ntrC* region of *A. vinelandii* (Toukdarian and Kennedy, 1986) (see Table 6:7). The results obtained indicated that sequences present within pCOS1 were able to correct the glutamine auxotrophy of these organisms but unable to correct their Ntr$^-$ phenotype. The inability of pCOS1 to correct the Ntr$^-$ phenotypes of ET8894 and UNF1848 may be due to a number of factors. For instance, the entire *ntrC* gene may not be present on pCOS1 (hybridization studies indicated a terminal position of the gene within the pCOS1 insert); if the gene is present, and is expressed, the gene product may not function in either of these hosts. In order to try and overcome any possible expression problem of the *ntrC* homologue within pCOS1 and to amplify the *ntrC* containing sequence, the 10 kb *Hin*dIII fragment was subcloned in the correct orientation (i.e. to make *glnA*$^+$) into the pDC100 *Hin*dIII dalation plasmid, pDC20. The resulting plasmid, pDC110, was then tested for its Ntr correcting ability in *E. coli* ET8894 and ET8556 (*ntrC* point mutant) by examining growth on M9 minimal media containing 0.2% (w/v) arginine as sole nitrogen source. The recombinant pDC110 did not correct the Ntr$^-$ phenotype of either of these mutant strains.

The results obtained from these studies indicate the presence of *ntrB* and *ntrC*-like genes within the *M. capsulatus* (Bath) genome. Although nucleotide sequence analysis of the *M. capsulatus* (Bath) *glnA* gene region showed the absence of any *ntr*-like sequences within the *glnA* 3' flanking region (see Chapter 5), these results confirm the presence of these genes and locate the *ntrC*-like gene to approximately 8.5 kb downstream of *glnA*.

6:2:6 Screening of Type I and Type II obligate methanotrophs for *glnA* and *ntrC* homologues.

Genomic DNA's from both Type I and Type II obligate methanotrophs were digested to completion with the restriction endonuclease *Eco*RI,

fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with either the 5' region of the *M. capsulatus* (Bath) *glnA* gene (*EpnI-SphI* 0.42 kb fragment of pDC1) or the *K. pneumoniae* *ntrC* gene (pMD114 1.5 kb *EcoRI-HindIII* fragment - see Figure 6:1). The resulting autoradiograph patterns are shown in Figures 6:13 and 6:14 respectively.

Specific *EcoRI* restriction fragments of both Type I and Type II methanotroph genomic DNAs exhibited a high degree of homology to the *M. capsulatus* (Bath) *glnA* probe. When the *K. pneumoniae* *ntrC* gene was used as a hybridization probe, both Type I and Type II methanotroph genomic DNA fragments exhibited homology to this probe. None of the *glnA* homologues corresponded in size to any *ntrC* homologues, with the exception of a 3.8 kb *Methylobacter capsulatus* Y *EcoRI* fragment, which exhibited homology to both probes.

6:2:7 Heterologous hybridization studies using the *glnB* genes of *E. coli* and *R. leguminosarum* as ~~~~ probes.

The pathways that regulate the expression of the *glnA* structural gene and the enzymatic activity of its product, both involve the P_{II} regulatory protein in a number of organisms. The structural gene for the P_{II} protein, *glnB*, has been identified and found to be located upstream of *glnA* in *A. brasilense*, *B. japonicum* and *R. leguminosarum* (Colonna-Romano *et al.*, 1987), a situation quite different from that in enteric bacteria (reviewed in Merrick, 1988(a)).

In order to determine the presence and location of *glnB* with respect to *glnA* in *M. capsulatus* (Bath), the *E. coli* and *R. leguminosarum* *glnB* genes were used as heterologous hybridization probes (the *EcoRI-SalI* fragment of pAH5 and the *EcoRI-BamHI* fragment of pAH3, respectively - see Figure 6:1).

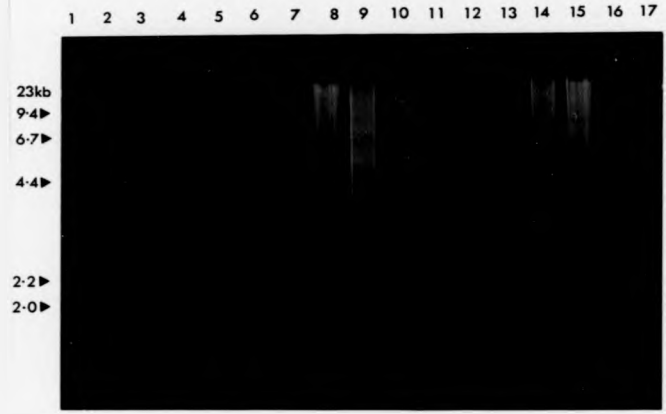
Figure 6.13 Screening of Type I and Type II obligate methanotroph genomic DNAs for the presence of *glnA* homologues by heterologous hybridisation.

Genomic DNAs from both Type I and Type II obligate methanotrophs were restricted to completion with the restriction endonuclease *Eco*RI, fractionated by agarose gel electrophoresis, Southern blotted and probed at low stringency (assuming an approximate 60% bp mismatch) with *M. capsulatus* (Bath) *glnA* (0.42 kb pDC1 *Kpn*I-*Sph*I fragment).

Key to tracks:

1 λ DNA restricted with *Hin*dIII
2 BG8 genomic DNA restricted with *Eco*RI
3 A20 genomic DNA restricted with *Eco*RI
4 S1 genomic DNA restricted with *Eco*RI
5 A4 genomic DNA restricted with *Eco*RI
6 *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
7 Y genomic DNA restricted with *Eco*RI
8 PM genomic DNA restricted with *Eco*RI
9 *M. rubra* genomic DNA restricted with *Eco*RI
10 5 genomic DNA restricted with *Eco*RI
11 12 genomic DNA restricted with *Eco*RI
12 PG genomic DNA restricted with *Eco*RI
13 OB3b genomic DNA restricted with *Eco*RI
14 OB5b genomic DNA restricted with *Eco*RI
15 OB4 genomic DNA restricted with *Eco*RI
16 OBBp genomic DNA restricted with *Eco*RI
17 λ DNA restricted with *Hin*dIII

Tracks 18 to 32 are autoradiographs of corresponding tracks 2 to 16 probed with the *M. capsulatus* (Bath) *glnA* gene probe.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23kb
9·4
6·7
4·4
2·2
2·0


18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
23kb
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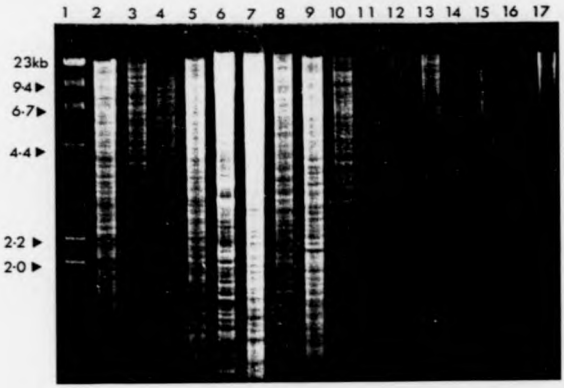
1·8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23kb
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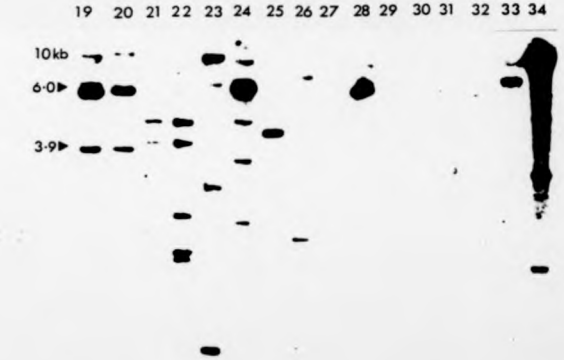
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10kb
6·0
3·9

Figure 4:14 Screening of Type I and Type II obligate methanotroph genomic DNAs for the presence of *ntrC* homologues by heterologous hybridisation.

Genomic DNAs from Type I, Type II obligate methanotrophs and *K. pneumoniae* were restricted to completion with the restriction endonuclease *Eco*RI, fractionated by agarose gel electrophoresis, Southern blotted and probed at low stringency (assuming an approximate 60% bp mismatch) with the *K. pneumoniae ntrC* gene probe (pMD114 1.5 kb *Eco*RI-*Hin*dIII fragment).

Key to tracks:

1 λ DNA restricted with *Hin*dIII
2 BG8 genomic DNA restricted with *Eco*RI
3 A20 genomic DNA restricted with *Eco*RI
4 S1 genomic DNA restricted with *Eco*RI
5 A4 genomic DNA restricted with *Eco*RI
6 PM genomic DNA restricted with *Eco*RI
7 *M. rubra* genomic DNA restricted with *Eco*RI
8 Y genomic DNA restricted with *Eco*RI
9 5 genomic DNA restricted with *Eco*RI
10 12 genomic DNA restricted with *Eco*RI
11 PG genomic DNA restricted with *Eco*RI
12 OB3b genomic DNA restricted with *Eco*RI
13 OB5b genomic DNA restricted with *Eco*RI
14 OB4 genomic DNA restricted with *Eco*RI
15 OBBp genomic DNA restricted with *Eco*RI
16 *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
17 *K. pneumoniae* genomic DNA restricted with *Eco*RI

Tracks 19 to 34 are autoradiographs of corresponding tracks 2 to 17 probed with *K. pneumoniae ntrC*.

M. capsulatus (Bath) genomic DNA was digested to completion with various restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with either ^{32}P-labelled *E. coli* or *R. leguminosarum glnB* gene probes. The resulting autoradiograph patterns are shown in Figure 6:15 and summarized on Table 6:8.

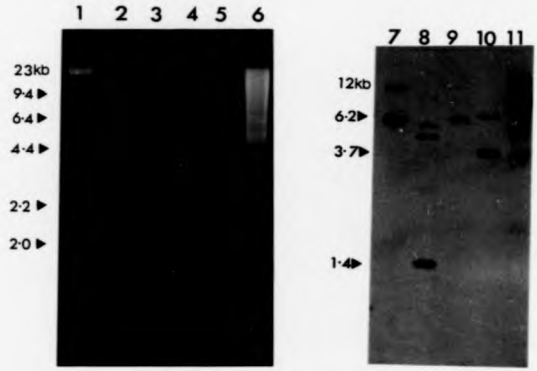
Specific *M. capsulatus* (Bath) restriction fragments exhibited homology to both *glnB* probes. *M. capsulatus* (Bath) restriction fragments of corresponding size were shown to hybridize to both *glnB* probes. Additional hybridizing fragments were observed when the *E. coli glnB* gene was used as hybridization probe. Specific *K. pneumoniae* *Eco*RI restriction fragments also exhibited homology to both *glnB* probes. None of the specific *M. capsulatus* (Bath) restriction fragments exhibiting homology to the *glnB* probes corresponded in size to any previously determined fragments upstream of the *M. capsulatus* (Bath) *glnA* gene (see Figure 6:11).

6:2:8 Heterologous hybridization studies with *K. pneumoniae* and *A. vinelandii ntrA* genes as heterologous hybridization probes.

M. capsulatus (Bath) genomic DNA was digested with a number of restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with ^{32}P-labelled *K. pneumoniae* and *A. vinelandii ntrA* genes (*Cla*I fragment from pMM17 and *Eco*RI-*Cla*I fragment of pAT705, respectively - see Figure 6:1). The resulting autoradiograph patterns are shown in Figures 6:16 and 6:17 respectively.

Specific *M. capsulatus* (Bath) restriction fragments exhibited a high degree of homology to both *ntrA* probes. The specific restriction fragments exhibiting homology to these probes were of corresponding size. Sequences present on pCOS1 did not hybridize to either of the *ntrA* probes used.

[A]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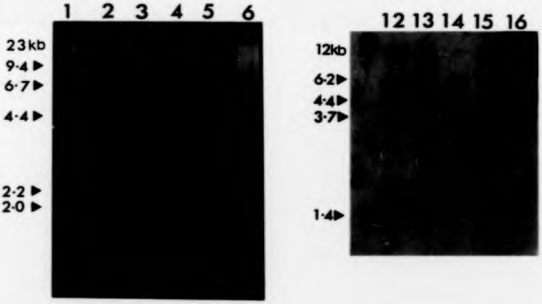
[B]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12 13 14 15 16
12kb
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4·4
3·7
1·4

Figure 6:13 Screening of *M. capsulatus* (Bath) genomic DNA for the presence of *glnB* homologues by heterologous hybridization.

Genomic DNAs from *M. capsulatus* (Bath) and *K. pneumoniae* were restricted with the restriction endonucleases indicated, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with *R. leguminosarum* or *E. coli glnB* gene probes at low stringency (assuming an approximate 70% bp mismatch).

Key to tracks:

[A] *R. leguminosarum glnB* probed

[B] *E. coli glnB* probed

1 λ DNA restricted with *HindIII*

2 *M. capsulatus* (Bath) genomic DNA restricted with *EcoRI*

3 *M. capsulatus* (Bath) genomic DNA restricted with *SalI*

4 *M. capsulatus* (Bath) genomic DNA restricted with *HindIII*

5 *M. capsulatus* (Bath) genomic DNA restricted with *PstI*

6 *K. pneumoniae* genomic DNA restricted with *EcoRI*

Tracks 7 to 11 and 12 to 16 are autoradiographs of corresponding tracks 2 to 6 probed with *R. leguminosarum glnB* (pAH3 0.4 kb *EcoRI-SalI* fragment) and *E. coli glnB* (pAH5 1.4 kb *EcoRI-BamHI* fragment) respectively.

Table 6:8 Sizes of *H. capsulatus* (Bath) genomic DNA fragments that exhibit homology to *E. coli* and *R. leguminosarum* *glnB* gene probes.

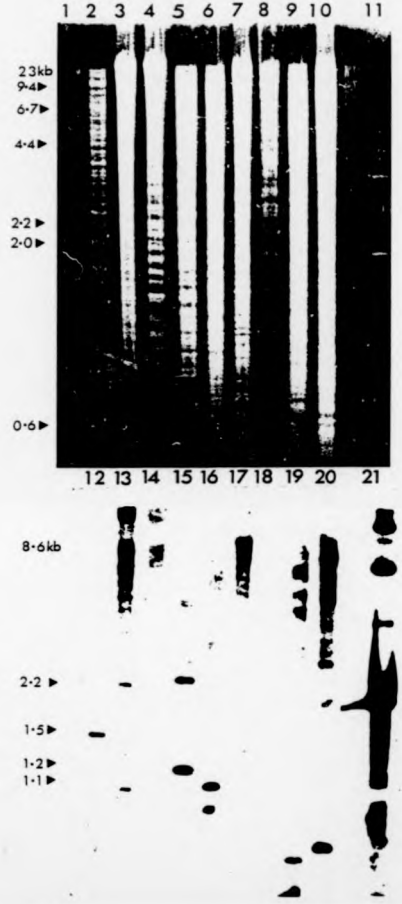
This table summarizes the data presented in Figure 6:15.

Homology with *R. leguminosarum glnB*

Restriction Endonuclease	*Eco*RI	*Sal*I	*Hin*dIII	*Pst*I	*K. pneumoniae* genomic *Eco*RI
Fragment size (kb)	12.0	5.6	6.2	6.6	19.0
	6.2	4.7		3.7	8.2
		1.4			

Homology with *E. coli glnB*

Restriction Endonuclease	*Eco*RI	*Sal*I	*Hin*dIII	*Pst*I	*K. pneumoniae* genomic *Eco*RI
Fragment size (kb)	12.0	9.4	6.2	8.6	19.0
	6.2	5.6	5.0	6.6	8.2
	4.4	4.7	4.1	5.0	4.2
	3.7	1.4		3.7	3.2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23kb
9·4
6·7
4·4
2·2
2·0
0·6
12 13 14 15 16 17 18 19 20 21
8·6kb
2·2
1·5
1·2
1·1

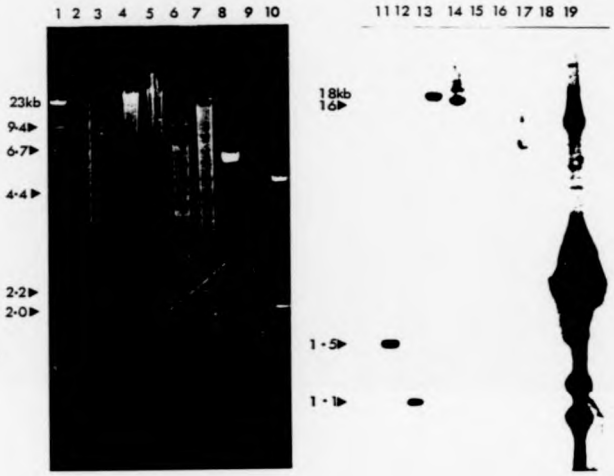
Figure 6:16 Screening of *M. capsulatus* (Bath) genomic DNA for the presence of *ntrA* homologues by heterologous hybridisation with *K. pneumoniae ntrA* as gene probe.

Genomic DNA from *M. capsulatus* (Bath) was restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at a stringency allowing approximately 40% bp mismatch with *K. pneumoniae ntrA* (1.9 kb *Cla*I fragment of pMM17).

Key to tracks:

1 λ DNA restricted with *Hin*dIII
2 *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
3 *M. capsulatus* (Bath) genomic DNA restricted with *Sal*I
4 *M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII
5 *M. capsulatus* (Bath) genomic DNA restricted with *Cla*I
6 *M. capsulatus* (Bath) genomic DNA restricted with *Sph*I
7 *M. capsulatus* (Bath) genomic DNA restricted with *Bam*HI
8 *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RV
9 *M. capsulatus* (Bath) genomic DNA restricted with *Pst*I
10 *M. capsulatus* (Bath) genomic DNA restricted with *Ava*I
11 pMM17 DNA restricted with *Cla*I

Tracks 12 to 21 are autoradiographs of corrsponding tracks 2 to 11 probed with *K. pneumoniae ntrA*.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11 12 13 14 15 16 17 18 19
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4·4
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18kb
16
1·5
1·1

Figure 6.17 Screening of *M. capsulatus* (Bath) genomic DNA for the presence of *ntrA* homologues by heterologous hybridization with *A. vinelandii ntrA* as gene probe.

M. capsulatus (Bath) genomic and pCOS1 DNAs were restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at a stringency allowing approximately 20% bp mismatch with *A. vinelandii ntrA* (pAT705 2 kb *Eco*RI-*Cla*I fragment).

Key to tracks:

1 λ DNA restricted with *Hin*dIII
2 *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI
3 *M. capsulatus* (Bath) genomic DNA restricted with *Sal*I
4 *M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII
5 *M. capsulatus* (Bath) genomic DNA restricted with *Kpn*I
6 *M. capsulatus* (Bath) genomic DNA restricted with *Pst*I
7 *M. capsulatus* (Bath) genomic DNA restricted with *Xho*I
8 pBR325 DNA restricted with *Eco*RI
9 pCOS1 DNA restricted with *Xho*I
10 pAT705 DNA restricted with *Eco*RI-*Hin*dIII

Tracks 11 to 19 are autoradiographs of corresponding tracks 2 to 10 probed with the *A. vinelandii ntrA* gene probe.

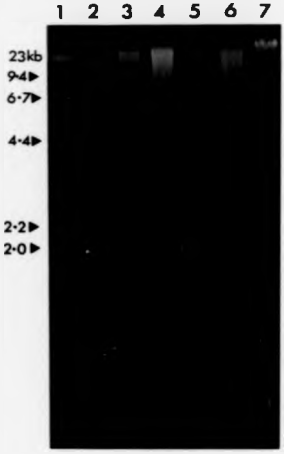
The *ntrA* gene product in enteric bacteria has been demonstrated to function as a novel form of RNA polymerase sigma factor (Hunt and Magasanik, 1985; Hirschman *et al.*, 1985). Studies of Merrick and Gibbins, (1985) revealed regions of homology between the abundant *E. coli* sigma factor, σ70 (RpoD) and *K. pneumoniae* NtrA. However, this homology was not reflected in hybridization studies carried out with *E. coli rpoD* and *K. pneumoniae ntrA* (Merrick and Stewart, 1985). The homology between *E. coli rpoD* and *M. capsulatus* (Bath) DNA sequences was thus determined by hybridization studies, in order to demonstrate the presence of any *rpoD*-like sequences and also show them to be distinct from *ntrA* homologues within the *M. capsulatus* (Bath) genome.

M. capsulatus (Bath) genomic DNA was digested with a number of restriction endonucleases, Southern blotted onto nitrocellulose and probed at low stringency with ^{32}P-labelled *E. coli rpoD* (*Hin*dIII fragment of pMM26 - see Figure 6:1). The resulting autoradiograph pattern is shown in Figure 6:18.

Specific *M. capsulatus* (Bath) restriction fragments exhibited homology to the *E. coli rpoD* gene probe, however, none of these fragments corresponded in size to the previously determined *ntrA* homologues (see Figures 6:16 and 6:17).

6:2:9 Screening of Type I and Type II obligate methanotrophs for *ntrA* homologues.

Genomic DNAs from Type I and Type II obligate methanotrophs were digested to completion with the restriction endonuclease *Eco*RI, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at low stringency with ^{32}P-labelled *A. vinelandii ntrA*. The resulting autoradiograph pattern is shown in Figure 6:19. Specific *Eco*RI genomic fragments of both Type I and Type II methanotrophs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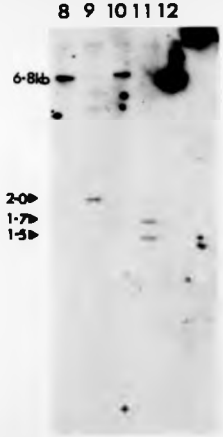
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6·8kb
2·0
1·7
1·5

Figure 4:10 Screening *M. capsulatus* (Bath) genomic DNA for the presence of *rpoD* (σ^{70}) homologues by heterologous hybridisation with *E. coli* *rpoD* as gene probe.

M. capsulatus (Bath) genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis, Southern blotted and probed at a stringency allowing approximately 60% bp mismatch with *E. coli rpoD* (pMM26 *Hin*dIII fragment).

Key to tracks:

1 λ DNA restricted with *Hin*dIII

2 *M. capsulatus* (Bath) genomic DNA restricted with *Eco*RI

3 *M. capsulatus* (Bath) genomic DNA restricted with *Sal*I

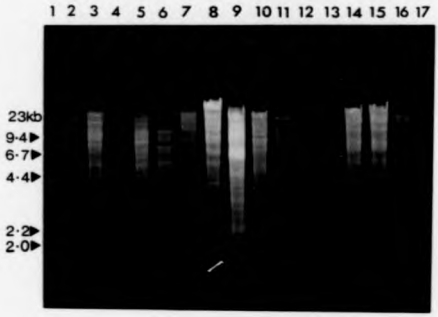
4 *M. capsulatus* (Bath) genomic DNA restricted with *Hin*dIII

5 *M. capsulatus* (Bath) genomic DNA restricted with *Pst*I

6 *M. capsulatus* (Bath) genomic DNA restricted with *Xho*I

7 *M. capsulatus* (Bath) genomic DNA restricted with *Bam*HI

Tracks 8 to 13 are autoradiographs of corresponding tracks 2 to 7 probed with *E. coli rpoD*.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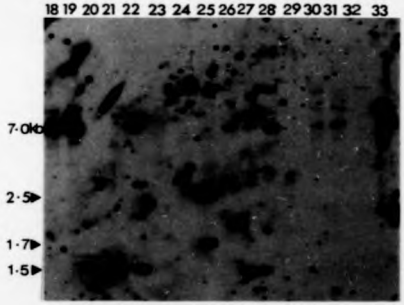
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7·0kb
2·5
1·7
1·5

Figure 4:19 Screening of Type I and Type II obligate methanotroph genomic DNAs for the presence of *ntrA* homologues by heterologous hybridization with *A. vinelandii ntrA* as gene probe.

Genomic DNAs from both Type I and Type II obligate methanotrophs were restricted with *Eco*RI, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at a stringency assuming an approximate 60% bp mismatch with *A. vinelandii ntrA* (pAT705 2 kb *Eco*RI-*Cla*I fragment).

Key to tracks:

1 λ DNA restricted with *Hin*dIII
2 BGB genomic DNA restricted with *Eco*RI
3 A20 genomic DNA restricted with *Eco*RI
4 S1 genomic DNA restricted with *Eco*RI
5 A4 genomic DNA restricted with *Eco*RI
6 Y genomic DNA restricted with *Eco*RI
7 *M. capsulatus* genomic DNA restricted with *Eco*RI
8 PM genomic DNA restricted with *Eco*RI
9 *M. rubra* genomic DNA restricted with *Eco*RI
10 5 genomic DNA restricted with *Eco*RI
11 12 genomic DNA restricted with *Eco*RI
12 PG genomic DNA restricted with *Eco*RI
13 OB3b genomic DNA restricted with *Eco*RI
14 OB5b genomic DNA restricted with *Eco*RI
15 OB4 genomic DNA restricted with *Eco*RI
16 OBBp genomic DNA restricted with *Eco*RI
17 λ DNA restricted with *Hin*dIII

Tracks 18 to 33 are autoradiographs of corresponding tracks 2 to 16 probed with *A. vinelandii ntrA*.

screened exhibited homology to the A. vinelandii ntrA gene probe.

6:2:10 Isolation of the *H. capsulatus* (Bath) *ntrA* structural gene.

Heterologous hybridization studies with the *K. pneumoniae* and *A. vinelandii ntrA* gene probes revealed a high degree of homology with specific *H. capsulatus* (Bath) sequences (see Section 6:2:8). As expression of *H. capsulatus* (Bath) DNA in *E. coli* and *K. pneumoniae* has been previously demonstrated (see Chapter 4), a 'shotgun' approach was taken in an attempt to isolate the *H. capsulatus* (Bath) *ntrA* structural gene.

H. capsulatus (Bath) genomic DNA was digested to completion with the restriction endonuclease *Hin*dIII, ligated to *Hin*dIII digested and dephosphorylated pBR325 and transformed into competent *E. coli* ET8045 (*ntrA* :: Tn10) and *K. pneumoniae* CK273 (*ntrA* 2273) cells. Selection for $NtrA^+$ transformants was made on M9 minimal agar containing chloramphenicol, tetracycline and 0.2% (w/v) arginine as sole nitrogen source for ET8045, and NFDM supplemented with histidine (25 $\mu g\ ml^{-1}$), chloramphenicol, ampicillin and 0.2% arginine as sole nitrogen source for CK273. No $NtrA^+$ transformants were isolated. The above procedure was also attempted with a *H. capsulatus* (Bath) pBR325 *Eco*RI gene library. Again, no $NtrA^+$ transformants were isolated.

These results suggested that either the complete gene had not been isolated from either library, or, if the complete gene had been isolated, and was expressed, that the *H. capsulatus* (Bath) *ntrA* gene product could not interact with *ntrC*-activated promoters of *E. coli* or *K. pneumoniae*.

6:3 Discussion.

Studies carried out by a number of workers have revealed that GS of enteric bacteria is regulated both at the level of transcription and post-

translationally. Post-translational regulation of GS activity is achieved by a complex cascade of events, ultimately controlled by the relative levels of glutamine and α-ketoglutarate in the cells (Ginsburg and Stadtman, 1973). Regulation of GS also occurs at the level of transcription such that, in nitrogen-limited cells the level of GS is ten-fold greater than that observed in cells grown with excess ammonia. The system which mediates this control is known as the nitrogen regulation (*ntr*) system, the function of which is ultimately regulated by the relative levels of glutamine and α-ketoglutarate (reviewed in Merrick, 1988(a)).

Previous studies on *M. capsulatus* (Bath) GS by Murrell and Dalton demonstrated the covalent modification of the GS enzyme, which was dependant upon the nitrogen status of the cell and also nitrogen-limited cells had GS levels six-fold greater than that observed in cells grown under nitrogen-excess conditions (Murrell and Dalton, 1983(b)). The observed increase in GS levels in nitrogen-limited *M. capsulatus* (Bath) cells may be due to increased expression of the GS structural gene, *glnA*. Regulation of *M. capsulatus* (Bath) *glnA* therefore, may be achieved by an analogous *ntr*-like system to that operating in the enteric bacteria. Merrick also tentatively proposed that in organisms where GS is subject to adenylylation, nitrogen control systems directly analogous to that in the enterics, may be found (Merrick, 1988(a)).

Previous studies have demonstrated adenylylation of GS in *M. capsulatus* (Bath) (Murrell and Dalton, 1983(c)) and results presented in Chapters 4 and 5 of this thesis have demonstrated, the requirement of the *E. coli ntr* system for regulated expression of the cloned *M. capsulatus* (Bath) *glnA* structural gene, the presence of a putative NtrC binding site and also a putative NtrA-dependant promoter sequence upstream of the *M. capsulatus* (Bath) *glnA* ORF. This data strongly suggested the presence of an analogous *ntr* system to that found in enteric bacteria, in *M. capsulatus* (Bath).

The results presented here confirm the existence of *ntr*-like genes in the *H. capsulatus* (Bath) genome. Heterologous hybridization studies with *K. pneumoniae* and *A. vinelandii* gene probes have revealed the presence of *ntrA*, *ntrB* and *ntrC* homologous sequences within the *H. capsulatus* (Bath) genome. In order to establish that these were legitimate *ntr* gene homologues, further heterologous hybridization studies were carried out. Previous studies have shown that the products of the *K. pneumoniae ntrC* and *nifA* genes exhibit considerable homology (Buikema *et al.*, 1985; *Drummond et al.*, 1986). Studies carried out in the identification of the *A. vinelandii ntrC* gene using the *K. pneumoniae ntrC* gene as hybridization probe, revealed common cross-hybridizing *A. vinelandii* genomic fragments with a *nifA*-like gene from *R. leguminosarum* (Toukdarian and Kennedy, 1986). Similar findings were made when the *K. pneumoniae nifA* gene was used to probe *Azorhizobium caulinodans* ORS571 genomic DNA (Pawlowski *et al.*, 1987). Results obtained in these studies using *K. pneumoniae nifA* as a hybridization probe against *H. capsulatus* (Bath) genomic digests, revealed no hybridizing fragments of corresponding size to those exhibiting homology to *K. pneumoniae ntrC*.

Similarly, studies carried out on NtrA from enteric bacteria have shown it to function as a novel RNA polymerase sigma factor (Hunt and Magasanik, 1985; Hirschman *et al.*, 1985) and when compared to other bacterial sigma factors, two regions of homology were identified (Merrick and Gibbins, 1985). Due to this homology, the gene encoding the most abundant *E. coli* sigma factor (σ70), *rpoD*, was used as a hybridization probe against *H. capsulatus* (Bath) genomic digests. Although specific *H. capsulatus* (Bath) genomic fragments exhibited homology to *rpoD*, none of these fragments corresponded in size to the genomic fragments exhibiting homology to either *K. pneumoniae* or *A. vinelandii ntrA* gene probes. In similar hybridization experiments carried out by Merrick and Stewart, between *E. coli rpoD* and *K. pneumoniae ntrA*, no homology between these

genes was detected (Marrick and Stewart, 1985).

Once the presence of putative *ntr* genes had been established within the *H. capsulatus* (Bath) genome, attempts to isolate the relevant sequences was undertaken. Screening of an *H. capsulatus* (Bath) cosmid library with the *K. pneumoniae ntrC* gene as hybridization probe, resulted in the isolation of pCOS1. Subsequant hybridization analysis of pCOS1, revealed the presence of the *glnA* structural gene some 8.5 kb upstream of the *ntrC* hybridizing region. In enteric bacteria (as well as a number of non-enteric bacteria), *ntrB* and *ntrC* are linked to *glnA* in the form of a complex operon, *ntrA* is unlinked to this region. In *H. capsulatus* (Bath) *ntrC* is not closely linked to *glnA* (supported by sequance data - Chapter 5), but lies some 8.5 kb downstream of *glnA*. This lack of *glnA-ntrC* linkage does not appear to be peculiar to *H. capsulatus* (Bath) as in *Rhizobium meliloti*, *Azorhizobium sesbaniae* ORS571 and *Rhodobacter capsulata*, linkage of *glnA* to *ntrC* has not been demonstrated (Szeto *et al.*, 1987; Pawlowski *et al.*, 1987; Hasselkorn, 1986).

The ability of pCOS1 and its derivative, pDC110 to restore an Ntr^+ phenotype to *K. coli* ET8894 and *K. pneumoniae* UNF1848 was not demonstrated. This inability of pCOS1 and pDC110 to restore an Ntr^+ phenotype to these organisms, may be due to only part of the putative *H. capsulatus* (Bath) *ntrC* structural gene being present within the pCOS1 and pDC110 inserts. The terminal position of the *ntrC* hybridizing region within pCOS1 and subsequent analysis of the hybridization data obtained, support this hypothesis. A 'chromosome walking' technique may be utilized in future experiments in order to isolate the entire *H. capsulatus* (Bath) *ntrC* gene region. Subsequant nucleotide sequencing of this region together with *in vivo* gene replacement studies, will enable the determination of the function of these genes within *H. capsulatus* (Bath).

Heterologous hybridization studies with the *ntrA* genes of *K. pneumoniae* and *A. vinelandii* as hybridization probes, revealed specific *H. capsulatus* (Bath) sequences exhibiting greater than 80% homology (according to our hybridization conditions - see Materials and Methods) to these gene probes. *H. capsulatus* (Bath) sequences present in pCOS1 did not exhibit homology to either *ntrA* probe, therefore *ntrA* in *H. capsulatus* (Bath) is unlinked to *glnA*. The *ntrA* structural gene has been cloned and sequenced from *K. pneumoniae*, *S. typhimurium*, *A. vinelandii* and *R. meliloti* and the predicted NtrA polypeptides are highly homologous (reviewed in Merrick, 1988(a)). The *ntrA* structural gene from these organisms are also unlinked to *glnA*. The high degree of homology exhibited by specific *H. capsulatus* (Bath) sequences to both *K. pneumoniae* and *A. vinelandii ntrA* gene probes, may reflect conservation of *ntrA* amongst bacteria. The isolation and subsequent nucleotide sequencing of *ntrA* from *H. capsulatus* (Bath) and other bacteria in the future, will reveal the degree of conservation of this novel RNA polymerase sigma factor.

Attempts to isolate the *ntrA* structural gene from *H. capsulatus* (Bath) *Hin*dIII and *Eco*RI genomic libraries by *in vivo* complementation of *E. coli* and *K. pneumoniae ntrA* mutants, failed to yield any $NtrA^{+}$ recombinants. A similar result was obtained by Pawlowski and colleagues when attempting to isolate the *A. sesbaniae* ORS571 *ntrA* gene using *E. coli ntrA* mutants (Pawlowski *et al.*, 1987). However, unlike *A. sesbaniae* ORS571, specific *H. capsulatus* (Bath) sequences exhibited a high degree of homology to the *ntrA* gene probes of *K. pneumoniae* and *A. vinelandii*. Therefore, the *H. capsulatus* (Bath) *ntrA* gene product is likely to function and hence complement both *E. coli* and *K. pneumoniae* mutants. Subsequent failure to isolate the *ntrA* structural gene may be due to the choice of restriction enzyme used in the construction of the gene libraries i.e. cleavage sites occurring within the structural gene or its control region. Alternatively, expression of *ntrA* in *E. coli* (deBruijn and Ausubel, 1983; Castano and

Bastarrachea, 1984), *K. pneumoniae* (Merrick and Stewart, 1985) and *A. vinelandii* (Merrick *et al.*, 1987) occurs constitutively, at a low level, independant of nitrogen status. Therefore, if expression of *ntrA* occurs in a similar manner in *M. capsulatus* (Bath), subsequent cloning may result in poor expression in the heterologous host, and therefore failure to complement the mutant *ntrA* allele present. Hence, future isolation of the *M. capsulatus* (Bath) *ntrA* structural gene may require a heterologous hybridization approach similar to that used in the isolation of the *M. capsulatus* (Bath) *glnA* structural gene (see Chapter 3).

The presence of *glnA*, *ntrA* and *ntrC* homologues in Type I and Type II obligate methanotrophs was determined. Specific *Eco*RI restriction fragments of all the Type I and Type II methanotrophs which were screened, exhibited homology to all three probes. The existence of *ntrA* and *ntrC* homologues in both Type I and Type II methanotrophs, as well as the Type X organism *M. capsulatus* (Bath), suggests that nitrogen metabolism in the methanotrophs is regulated by a global *ntr* system. Previous studies on ammonia assimilation in the methanotrophs by Murrell and Dalton, revealed that the amount of GS enzyme in the Type II methanotrophs did not vary significantly with the nitrogen status of the cell. Contrastingly, in the Type I methanotrophs, the amount of GS enzyme appeared to be regulated by nitrogen status (Murrell and Dalton, 1983(b)).

Therefore, the presence of *ntr* homologues in the methanotrophs may suggest a role in the regulation of *glnA* in Type I organisms and a possible role in nitrogen fixation (*nif*) regulation in Type II organisms. The apparent lack of *glnA* regulation in Type II methanotrophs may be due to a requirement of GS for ammonia assimilation under all growth conditions, as ammonia is assimilated exclusively by GS-GOGAT in these organisms (Murrell and Dalton, 1983(b)). Similar findings have been reported from studies carried out on *A. vinelandii*. The presence, therefore, of *ntr* homologues in Type II methanotrophs may reflect a requirement, for regulation of the

genes encoding nitrogen fixation capability and/or, for growth on poor nitrogen sources.

In a recent review by Merrick, it was proposed that a possible link may exist between organisms which possess, a GS enzyme which is subject to adenylylation and a nitrogen control system directly analogous to that in enteric bacteria. In such organisms, *glnB* and *glnD* gene homologues may also be expected (Merrick, 1988(a)). As mentioned earlier, studies carried out on purified *H. capsulatus* (Bath) GS have revealed this enzyme to be subject to adenylylation (Murrell and Dalton, 1983(c)) and results already presented in this thesis have revealed the presence of *ntrC* gene homologues in this organism. The presence of *glnB* homologues in *H. capsulatus* (Bath) has also been determined by heterologous hybridization studies carried out with the cloned *glnB* genes from *E. coli* (Stauffer *et al.*, 1981) and *R. leguminosarum* (Colonna-Romano *et al.*, 1987) as hybridization probes. Specific *H. capsulatus* (Bath) genomic fragments of corresponding size exhibited homology to both *glnB* gene probes, indicating the presence of a *glnB* homologue in the *H. capsulatus* (Bath) genome. Additional *H. capsulatus* (Bath) hybridizing fragments exhibiting homology to the *E. coli glnB* gene probe may be due to the presence of extra upstream sequences present on the *E. coli glnB* probe. Interestingly, *R. leguminosarum glnB* has been reported to not cross hybridize with either *K. pneumoniae* or *E. coli glnB* (Merrick - personal communication). However, these studies have revealed specific *K. pneumoniae* genomic fragments exhibiting homology to the *R. leguminosarum glnB* gene probe, when hybridization was carried out at low stringency.

The results obtained from these studies on *H. capsulatus* (Bath) add increasing support therefore to the hypothesis of Merrick (Merrick, 1988(a)). However, studies are still required on a wider range of organisms to validate this hypothesis.

CHAPTER 7

Genetic transformation of *M. capsulatus* (Bath)

7:1 Introduction.

One of the reasons for the slow progress in the application of molecular genetics to the study of the biology of 'exotic' microorganisms is often that DNA cannot be introduced into them by any of the usual methods (transformation, transduction, conjugation or protoplast fusions).

The lack of efficient gene transfer systems has been a major hurdle in attempts to study genetics in obligate methanotrophs. Therefore, these studies were undertaken in an attempt to develop a method of genetic transformation in the Type X obligate methanotroph, *M. capsulatus* (Bath).

7:2 Results.

7:2:1 Antibiotic sensitivity of *M. capsulatus* (Bath).

In order to determine potential vectors for use in subsequent transformation experiments, an antibiotic sensitivity spectrum of *M. capsulatus* (Bath) was determined. The stability of these antibiotics at 45°C (the optimum growth temperature of *M. capsulatus* (Bath)) for prolonged periods (up to 2 weeks) was also assessed.

Antibiotic multidisks (Oxoid and Mastring) were placed onto freshly prepared *M. capsulatus* (Bath) lawns and incubated under an atmosphere of CH_4 + air (50 : 50 v/v). Table 7:1 shows the effect of each antibiotic on the *M. capsulatus* (Bath) lawn after a 2 week exposure.

Of the 19 antibiotics tested, only 3 (kanamycin, gentamycin and streptomycin) appeared to be stable at 45°C for a prolonged period and

Table 7:1 Antibiotic sensitivity profile of *M. capsulatus* (Bath).

Antibiotic	Concentration/disk	sensitivity
Gentamycin	10 µg	+++
Colistin sulphate	10 µg	-
Nitrofurantoin sulphate	200 µg	-
Sulphafurazole	500 µg	-
Kanamycin	30 µg	+++
Ampicillin	25 µg	-
Co-trimoxazole	25 µg	-
Tetracycline	50 µg	-
Cephaloridine	5 µg	-
Erythromycin	5 µg	++*
Cloxacillin	5 µg	-
Novobiocin	5 µg	-
Lincomycin	2 µg	-
Penicillin G	1 unit	-
Chloramphenicol	25 µg	-
Streptomycin	10 µg	+++
Sulphatriad	200 µg	-
Oleandomycin	5 µg	-

* = High frequency of spontaneous resistance.

+ = Degree of sensitivity determined by size of zone of inhibition around each disk, +++ being very sensitive, to + least sensitive. - No detectable effect.

prevent growth of *M. capsulatus* (Bath). Kanamycin (10 $\mu g\ ml^{-1}$) and streptomycin (20 $\mu g\ ml^{-1}$), when used in liquid cultures of *M. capsulatus* (Bath) also prevented growth of the organism over a 2 week period. Ampicillin (50 $\mu g\ ml^{-1}$) and tetracycline (10 $\mu g\ ml^{-1}$) contrastingly prevented growth of *M. capsulatus* (Bath) in batch culture for approximately 72 hours at 45°C after which time normal growth was observed. These results enabled the determination of choice of plasmid vectors for use in transformation experiments. Broad-host range plasmids conferring either kanamycin resistance or streptomycin resistance were used in these experiments.

7.2.2 Development of a transformation system for *M. capsulatus* (Bath).

Induction of competence of *M. capsulatus* (Bath) was attempted using five published methods (Maniatis *et al.*, 1982; Hanahan, 1983; Klebe *et al.*, 1983, Merrick *et al.*, 1987; Dower, 1987). Transformation experiments were carried out with CsCl-purified pVK100, pSF6, pRK2501, pKT231 and pGSS33 (see Chapter 2, Table 2:2). These plasmids were chosen, as plasmids of groups IncP, Q and W have the ability to be stably maintained in a wide range of Gram-negative bacteria. Derivatives of these plasmids have been successfully transferred and maintained in a number of methylotrophs and obligate methanotrophs (Toukdarian and Kennedy, 1984; McFheat *et al.*, 1987; Al-Taho and Warner, 1987; Jayaseelan and Guest, 1979; Warner *et al.*, 1980; Hennam *et al.*, 1982; Moore *et al.*, 1983; Allen and Hanson, 1985).

Despite alteration of various parameters such as plasmid DNA concentration (0.25 → 5 $\mu g/ml$), heat shock temperature (37°C → 50°C) and exposure time (2-5 minutes) as well as length of expression time (2-18 hours) for each of the five methods, none of these methods yielded transformant colonies. Control experiments with *E. coli* HB101, yielded between 10^2 to 10^4 transformants per μg of chosen plasmid (pVK100, PSF6,

pRK2501, pKT231 or pGSS33) for all five methods.

7:2:3 Deoxyribonuclease activity in *M. capsulatus* (Bath).

A number of factors can strongly affect plasmid transformation of bacteria. Amongst these factors, the cellular content of nucleases may impair transformation, in as much as degradation of plasmid DNA can result in a decrease of the transformation efficiency. Hence, due to the lack of success in producing transformant colonies with the aforementioned methods an analysis of the content of DNases of *M. capsulatus* (Bath) was carried out as described by Rama *et al.*, 1987. Using this method, no DNase activity was detected in both whole cells or cell extracts of *M. capsulatus* (Bath).

7:2:4 Marker exchange mutagenesis.

The generation of a *M. capsulatus* (Bath) *glnA* mutant by the *in vivo* gene replacement technique (marker exchange) (Ruvkun and Ausubel, 1981) was undertaken, using the method described by Toukdarian and Lidstrom, (1984).

A single three-way mating between *E. coli* HB101 harbouring the *M. capsulatus* (Bath) *glnA* :: Ω recombinant pDC20, *E. coli* HB101 harbouring pRK2013 and *M. capsulatus* (Bath) was carried out essentially as described in Toukdarian and Lidstrom (1984).

No GlnA$^-$, streptomycin resistant *M. capsulatus* (Bath) transconjugants were isolated after three weeks incubation. However, only a single mating was attempted, and further attempts are required in order to determine the viability of this method for the generation of mutants in *M. capstulatus* (Bath).

7:3 Discussion.

These studies were initiated to develop a genetic transformation system for *M. capsulatus* (Bath). The only previous report of genetic transformation in an obligate methanotroph was by Williams and Bainbridge (1977), who transformed *M. capsulatus* (Foster and Davis strain) with linear DNA. Large amounts of DNA (100 $\mu g\ ml^{-1}$) were however, required, and no report to date has been made of plasmid transformation in these organisms.

A transformation system is an essential tool for the introduction of non-conjugative plasmids into recipient strains. An efficient transformation method facilitates the introduction of recombinant DNA molecules, into the strain of interest. Results from these studies parallel previous attempts to transform these organisms (Lidstrom-O'Connor, Pers. Comm.). Although none of these published methods proved successful with *M. capsulatus* (Bath), they have proved successful with a wide range of Gram-negative bacteria (Hinton, 1986).

Recently, the technique of electro-transformation (electroporation) has revolutionized the transformation of bacteria. Electroporation - the formation of holes or pores in the cell membrane by high voltage electric shock - has been used successfully to give very high transformation frequencies to a wide range of bacterial genera previously thought untransformable. A number of recent reviews cover the applications of electroporation and its successes in the transformation of a number of bacterial genera (Shigekawa and Dower, 1988; Chassy *et al.*, 1988; Potter, 1988). The successful application of electroporation to the transformation of a number of bacterial species suggests that the technique may be suitable for transformation of a range of bacterial species although the optimal conditions will probably have to be carefully determined in each case.

Although the conditions for electroporation of *E. coli* (Dower, 1987) using the BioRad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) did not result in the transformation of *M. capsulatus* (Bath), further experimentation is required in order to determine the viability of this method for transformation of this organism. Variables affecting transformation frequency with this method may include the shape and duration of the pulse, initial field strength, the number of discharges applied, the physiological condition of the cells and also the electroporation buffer used. For example, the field strength (6.25 kV cm^{-1}) used in these studies may not be great enough for transformation of *M. capsulatus* (Bath) (c.f. *Bordetella pertussis* requires 25 kV cm^{-1} for optimal frequency of transformation! - field strength was found to be the most important variable in these studies (Zealey *et al.*, 1988)).

The advances made in the manipulation of genetic material from *M. capsulatus* (Bath) during the course of these studies now requires an efficient gene transfer and a high frequency transformation system for this organism. None of the methods tested in these studies resulted in the isolation of transformants. However, electroporation is now the fastest and simplest method for transforming bacteria and its increasing success rate in transforming a wide range of organisms, has the greatest potential for success in *M. capsulatus* (Bath).

CHAPTER 8

General Overview.

The studies outlined in this thesis were initiated to develop techniques of genetic manipulation in the obligate methanotrophs. Ammonia assimilation was chosen for study for a number of reasons; a) The molecular genetics of ammonia assimilation has been extensively studied in the Enterobacteriaceae. Therefore, due to the availability of gene probes, mutants, etc. it was an ideal candidate for use as a model system; b) The physiology and biochemistry of ammonia assimilation has been studied in detail in obligate methanotrophs, especially in *M. capsulatus* (Bath); c) Information regarding regulation of nitrogen metabolism in organisms other than the Enterobacteriaceae has recently been accummulating. Therefore, information gleaned from these studies will contribute to the pool of knowledge thus far accrued and help determine the mechanisms by which microorganisms are able to co-ordinate nitrogen assimilation.

Using a heterologous hybridization approach, the structural gene for glutamine synthetase (*glnA*), an enzyme which plays a central role in nitrogen assimilation, was cloned on a 5.2 kb *M. capsulatus* (Bath) *Eco*RI genomic DNA fragment. An internal *glnA* gene fragment from *K. pneumoniae* M5al was used as a gene probe to isolate the *M. capsulatus* (Bath) *glnA* recombinant. Heterologous hybridization studies carried out using a cloned *Anabaena* 7120 *glnA* gene probe indicated a lack of detectable homology to any *M. capsulatus* (Bath) sequences, however, subsequent sequencing of the *M. capsulatus* (Bath) *glnA* structural gene revealed a 59% similarity at the nucleotide level with the *Anabaena* 7120 *glnA* gene. Therefore, caution must be observed in the interpretation of hybridization data.

Expression studies on the cloned *M. capsulatus* (Bath) *glnA* structural gene revealed the cloned gene to carry its own control region and that transcription and translation of the gene occurred in the heterologous hosts *E. coli* and *K. pneumoniae*, to complement the glutamine auxotrophy of these organisms. This is the first report of the expression of an obligate methanotroph gene producing an active protein in a heterologous host. Regulatory studies carried out in Ntr^+ and Ntr^- heterologous hosts under nitrogen-limiting and nitrogen-sufficient conditions revealed a requirement for a functional Ntr system for regulated expression of the *M. capsulatus* (Bath) *glnA* gene. Further studies are required in a variety of strain backgrounds to further elucidate the nature of regulated expression of the *M. capsulatus* (Bath) *glnA* structural gene.

The size of the *M. capsulatus* (Bath) GS subunit was determined as M_r 60,000 by *in vitro* and *in vivo* expression systems. Additionally, the recombinant pDC2 directed the synthesis of a M_r 42,000 polypeptide, thought to be the result of a fusion between part of the chloramphenicol acetyl transferase gene of the vector and a gene carried in the cloned fragment. Further studies are required to elucidate the origins and nature of this polypeptide.

The nucleotide sequence of the *M. capsulatus* (Bath) *glnA* structural gene revealed the *glnA* coding region to comprise of 1407 bp encoding a polypeptide of M_r 51,717 consisting of 468 amino acids. On the basis of these results, contrary to the suggested decameric structure of the *M. capsulatus* (Bath) GS enzyme (Murrell and Dalton, 1983(c)), it is proposed that the *M. capsulatus* (Bath) GS enzyme is dodecameric in nature as found in most other reported prokaryotic GS enzymes.

The codon usage pattern of the *M. capsulatus glnA* gene reflects the high reported G + C content of this organism and comparison of the patterns of codon usage of *glnA* genes from *E. coli*, *S. typhimurium*, *T. ferrooxidans*, *Anabaena* 7120 and *M. capsulatus* (Bath) suggest that *M. capsulatus* (Bath)

DNA would be efficiently translated in the majority of these organisms.

The nucleotide sequence and nucleotide sequence derived GS amino acid sequence, when compared to a variety of prokaryotic GS sequences revealed a high degree of conservation of *glnA* and the GS polypeptide.

Comparison of prokaryotic and eukaryotic GS amino acid sequences also showed a high degree of conservation of the GS polypeptide. This conservation occurred mainly in five regions (all associated with the proposed active site). These five regions were also highly conserved in the *N. capsulatus* (Bath) GS enzyme.

Analysis of the regions surrounding the *N. capsulatus* (Bath) *glnA* coding region revealed the existence of three putative promoter elements upstream of the *glnA* coding region. Two promoters resembling the *glnAp1*-type promoter of enteric organisms were found located on either side of a promoter element resembling the NtrA dependent promoter *glnAp2*. In enteric organisms, the *glnAp1* promoter lies approximately 100 bp upstream of the NtrA dependent promoter *glnAp2*. In *N. capsulatus* (Bath) the *glnA* gene also appears to be regulated by promoters resembling *glnAp1* and *glnAp2*. However, the relative positions of these putative promoters and the putative NtrC binding site differ from that found in the *glnA* gene leader regions of enteric bacteria.

Although the mechanism of *glnA* regulation in enteric organisms is fairly well documented, further studies are required to elucidate the nature of *glnA* regulation in *N. capsulatus* (Bath). Primary studies have suggested the requirement of a functional Ntr system for regulated expression of *N. capsulatus* (Bath) *glnA* in *E. coli*. Heterologous hybridization studies have revealed the presence of *ntrA*, *ntrB*, *ntrC* as well as *glnB* homologues in the *N. capsulatus* (Bath) genome, thereby giving increasing support to the idea of the presence of an analogous nitrogen regulatory system in *N. capsulatus* (Bath).

Analysis of the 3'-flanking region of the *N. capsulatus* (Bath) *glnA* gene revealed the existence of a small ORF encoding a polypeptide of M_r 7022. The function of this polypeptide encoded by this ORF remains to be elucidated. In enteric bacteria (as well as a number of non-enteric bacteria) the *ntrB* and *ntrC* genes lie directly downstream of *glnA*. In *N. capsulatus* (Bath), nucleotide sequence analysis and heterologous hybridization analysis has revealed *ntrB* and *ntrC* to be unlinked to *glnA* and lie some 8.5 kb downstream of *glnA*. Subsequent cloning of the *N. capsulatus* (Bath) *ntrB*, *ntrC* (partially cloned on pCOS1) and *ntrA* genes will enable the elucidation of the role of these genes in the regulation of nitrogen metabolism in *N. capsulatus* (Bath).

The development of an efficient gene transfer and transformation system in *N. capsulatus* (Bath) is now vitally important for the introduction of constructs into *N. capsulatus* (Bath) and to produce mutants with selectable markers by marker exchange. Attempts at developing a transformation method in these studies have failed using reported transformation protocols. However, with the advent of electrotransformation, this method offers the greatest chance of success at transforming this currently untransformable bacterium and in turn aid the advancement of obligate methanotroph genetics.

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